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THE UNIVERSITY OF ALBERTA

IMPROVEMENT OF CANOLA SAUCE QUALITY WITH PURE CULTURE INOCULATION OF MOROMI

by

Ⓒ **Araba A. Coleman**

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF **Master of Science**

IN

Food Processing

Department of Food Science

EDMONTON, ALBERTA

Fall 1987

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled **IMPROVEMENT OF CANOLA SAUCE QUALITY WITH PURE CULTURE INOCULATION OF MOROMI** submitted by **Araba A. Coleman** in partial fulfilment of the requirements for the degree of Master of Science in Food Processing.

....

Supervisor

Monica Palcic

Michael E. Stiles

Ronald Whitehouse

Date: 30-04-87

DEDICATION

Dedicated to

My Beloved Family

ABSTRACT

Canola sauce produced through a short fermentation employing *Aspergillus oryzae* and *A. sojae* lacked some organoleptic quality associated with high quality soy sauce. This was due to the absence of some secondary microbial fermentations in the moromi stage. These secondary fermentations involve bacteria and yeasts which are able to establish themselves in the moromi during long natural fermentation.

To improve the sauce quality, *Pediococcus halophilus*, *Saccharomyces rouxii* and *Torulopsis versatilis*, considered the most important microorganisms in the secondary fermentation of soy sauce, were deliberately introduced into moromi of canola sauce. They were first 'trained' to grow in media containing 18% w/v NaCl before inoculation.

Transfers of the microorganisms from their basic media into high salinity media involved prolonged lag phases, which were restored to normal after subsequent subculturing in fresh high salinity media. After reaching cell counts of 10^7-10^8 CFU/mL, they were inoculated into the canola mash for fermentation, which lasted 31 d. One fermentation procedure involved the stepwise inoculation of *P. halophilus* at the beginning, followed by *S. rouxii* and *T. versatilis* after 4 days. The other procedure involved simultaneous (all-in) inoculation of the three microorganisms at the start of fermentation. A batch with no inoculation served as control.

The deliberately fermented canola sauces involving the stepwise and simultaneous inoculations resulted in final products which had chemical qualities generally similar to those of commercial Kikkoman sauce. The compositional analyses of the deliberately fermented sauces yielded 1.2% w/v total soluble nitrogen (TSN), 0.40-0.41%w/v amino nitrogen, a nitrogen yield of 67.19%, 1.60-1.88% w/v lactic acid, and a total titratable acidity (TTA) value of 6.75-10.19 meq/100 mL sauce. The glucose content ranged between 0.75-1.56% w/v, while 0.13-0.20% w/v sucrose was obtained in the fermented sauces. An ethanol concentration of 1.5-1.6% w/v was found in the canola sauces, and the final salt content ranged between 17.46-17.52% w/v. Most of these results compared favourably to the commercial Kikkoman sauce and to literature values.

Glutamic acid, the most important amino acid in soy sauce, was found to be higher in the microbial fermented canola sauces than in Kikkoman sauce. Glutamic acid ranging from 49.62-53.32 μmole/mL was found in canola sauces, while only 42.00 μmole/mL was found in the soy sauce.

The concentrations of most of the organic acids in the canola sauces, especially in the all-in inoculated sauce, were greater than those in the soy sauce.

The lactic acid content in the canola sauces indicated excessive lactic fermentation. Concentrations of about 1543-1880 mg/100 mL sauce were recorded for the canola sauces, as compared to about 336-500 mg/100 mL for the soy sauce. Except for a high amount of formic acid in the all-in inoculated sauce, 70 mg/100 mL as compared to 48.14 mg/100 mL in the stepwise inoculated canola sauce and 63.7 mg/100 mL in the commercial soy sauce, no other undesirable acid was detected in the sauces.

The absence of some characteristic soy sauce aromas in the canola sauces and the inconsistency in sensory evaluation of the products by untrained panelists were factors in the canola sauces being scored lower than the commercial Kikkoman sauce.

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1. INTRODUCTION

Techniques of enzymatically hydrolysing certain protein foods into amino acids and small peptides, to make them more nourishing and flavourful, have long been known in the Orient (Yokotsuka, 1986). Amongst the protein foods so prepared by fermentation are soybeans.

Soy sauce is a popular liquid condiment produced by fermenting soybeans and wheat mixtures in the presence of salt. This condiment is consumed daily in the Orient.

Its primary role in the Japanese diet is as a source of salt, flavour and colour for their bland and basic diet such as rice and boiled vegetables. Soy sauce contributes 2.4 g out of 80 g of the daily protein intake in Japan; as such it has little significance as a source of protein or amino acids (Bureau of Foods, Japan, 1976: as cited by Yokotsuka, 1986).

Soy sauce has gained commercial potential in the West, mainly because of the great popularity which the Oriental cuisine (restaurant) has enjoyed recently. Fukushima (1985) reported that the production of fermented soy sauce in the United States has averaged a 15% increase annually for the 10 years prior to his review, and it has become widely acceptable to the Caucasian people in the U.S.

Unlike the U.S., Canada has no manufacturing plant and relies on imports of the sauce from Hong Kong, Japan, Korean Republic and Singapore (Wood, 1982). A major ingredient of the sauce is soybeans, which grow only in certain parts of Canada. On the other hand, wheat is widely grown and is a major Canadian crop. Therefore, a manufacturing plant would depend heavily on the availability of these raw materials. Soybeans, as indicated, are not a major crop of Canada, so to substitute for this raw material in sauce production, one must consider the availability of other protein-rich crops and, more importantly, their prices relative to that of soybeans.

Researchers have come up with a protein-rich oil seed from the rapeseed family. Usually referred to in Canada as "canola", it is a source of edible oil for humans and the protein-rich meal is fed to livestock and poultry. It is known as the "Cinderella Crop" of Canada and, unlike other rapeseed crops, it has little or no erucic acid and the glucosinolate content is very low.

Canada is one of the major rapeseed producing countries in the world but, currently, canola is produced only in Canada.

Canola supplies about 46% of the vegetable oil in the diet of Canadians (Biely and Salmon, 1981). On the average, the protein-rich meal obtained after oil extraction contains 37-38% protein and, depending on the cultivar, the protein could be as low as 35% and as high as 40% (Clandinin *et al.*, 1981).

Protein quality of canola meal, as determined by Campbell and Eggum (1980), compared favourably with other protein products such as high quality fish meal. The biological value (BV), which compares essential amino acids present in protein of canola meal to amino acid requirements of a growing animal, was found to be in excess of 90%. The net protein utilization (NPU) was found to have an average value of 76%. Campbell and Eggum (1980) also reported that individual amino acids present in meal were well digested and indicated a value of 88% as the digestibility coefficient. Sosulski and Sarwar (1973) rated canola meal as the best vegetable protein.

In 1980, Ooraikul *et al.* attempted to prove the feasibility of substituting canola meal for soybean meal in sauce production. The rapeseed sauces produced compared well with the commercial sauce. The success of the preliminary studies involving canola meal and wheat in sauce production led to further research. An efficient technique and, more importantly, a relatively cheap process was investigated. Ma (1985) was able to produce the sauce within 5 weeks by using an endoprotease to effectively break down the crude proteins present in canola meal (CM). Thus, maximum proteolysis occurred when the hydrolysed meal and wheat mixture was inoculated with *Aspergillus* cultures for sauce production. Ma (1985) also reported that the greatest deficiencies in the quality of the canola sauce produced were its low acidity and lack of aroma. These deficiencies were attributed to the inadequacy of acid and alcohol fermentations, and contributed to the lower quality of the canola sauce when compared to Kikkoman sauce.

In order to provide a greater understanding of these deficiencies, as well as to rectify them to improve the sauce quality, further research was necessary. It has been shown that yeasts and bacteria, such as *Saccharomyces rouxii*, *Pediococcus halophilus*, *Lactobacillus delbrueckii* and *Torulopsis* species, are involved in the secondary (moromi) fermentation of soy sauce (Yokotsuka, 1960, 1985, 1986; Fukushima, 1979, 1985; Yong and Wood, 1976; Hesseltine and Wang, 1972).

Under appropriate environmental conditions, these organisms have been associated with the production of organic acids, alcohols and some other desirable volatile compounds during the fermentation (Yokotsuka, 1986; Fukushima, 1985). The research described in this thesis involves a deliberate introduction of some of these microorganisms into the moromi of canola sauce, either in combination or sequentially.

The microorganisms were first "trained" to tolerate 18% salt concentration in growth media, before inoculation into the moromi. Their growth activities were monitored throughout the 5 week fermentation period by taking samples of the moromi at appropriate time intervals to analyse for some of their metabolites. The resultant sauces were then compared chemically and organoleptically with uninoculated (control) canola sauce and Kikkoman sauce.

2. LITERATURE REVIEW

2.1 Soy Sauce

2.1.1 Historical aspects

Shoyu or soy sauce, a representative protein food, produced in large quantities in the Orient, has had an extraordinarily long history. It is basically a liquid food condiment, prepared from field crops, through fermentation.

A dark brownish liquid with a distinct pleasant aroma, it is used widely for adding flavour to a great many foods, such as meat, poultry and fish, and for barbeque and other sauces, for flavouring cooked vegetables, and for seasoning in general (Hesseltine, 1965).

This historical brewing of soy sauce goes back many centuries in countries like China and Japan. Originating in China about 2,500 years ago, it was introduced in Japan alongside a change to vegetable diet by Chinese Buddhist priests in the 6th Century (Hesseltine, 1965; Fukushima, 1985; Yokotsuka, 1985). The original product was transformed into the present day Japanese soy sauce by the 17th Century (Fukushima, 1985). Since the introduction in Japan, other Asian countries and even the United States have carried out the fermentation of soy sauce.

The manufacturing process has always involved the growth of mould on a substrate. Originally, the substrate was fish or meat, but this was substituted with soybeans before the introduction of the sauce into Japan (Fukushima, 1985). Presently, in the regular Japanese soy sauce, wheat is used with an equal amount of soybeans and the characteristic aroma is developed from the wheat (Fukushima, 1985).

2.1.2 Types and varieties

In Japan, two basic groups or types of soy sauces are recognised. These two groups are the fermented soy sauce and the chemical soy sauce. Though the genuinely fermented sauce has had a long history as human food, the chemical sauce's history is only several decades long (Fukushima, 1985).

Traditionally, the proteins and carbohydrates present in the raw materials for production of the sauce are slowly hydrolysed under mild conditions (below 30°C) for a minimum period of 6 months to produce the fermented sauce. Unlike the "genuine" sauce, the chemical sauce is produced by a quick HCl-hydrolysis of raw material components under severe conditions (Fukushima, 1985), producing a cheap sauce with undesirable compounds. In an effort to improve upon the chemical soy sauce, a semi-chemical sauce was devised. Though yeast fermentation takes place after the initial hydrolysis of the soybeans by a low concentration of HCl (7-8%), in the presence of wheat koji, the resultant sauce still has the undesirable compounds but with improved odour and flavour.

The Japan Agricultural Standard (JAS) recognises five varieties of soy sauce, each of which is classified into three grades, namely Special, Upper and Standard. Grading is determined by organoleptic evaluation, contents of total nitrogen, alcohol, soluble solids other than sodium chloride, and colour (Fukushima, 1985). High quality sauce is assigned special grade as it is only made by action of microorganisms. As such, chemical and/or enzymatic hydrolysates are not considered for special grade soy sauce. Table 2.1 shows the annual production of grades of soy sauce (Fukushima, 1985).

The five varieties mentioned above include Koikuchi-shoyu, Usukuchi-shoyu, Tamari-shoyu, Shiro-shoyu and Saishikomi-shoyu. Typical compositions of these varieties of soy sauce are shown in Table 2.2.

Koikuchi-shoyu is the regular soy sauce and the most representative Japanese fermented sauce. Totalling about 85% of soy sauce consumed in Japan (Yokotsuka, 1985), it is characterized by a strong aroma, myriad flavour and deep-reddish brown colour (Fukushima, 1985). The sauce is made from approximately equal amounts of wheat and soybeans. The koikuchi mash is subjected to vigorous lactic and alcoholic fermentations and the finished product is pasteurized at about 80°C, giving the characteristic reddish-brown colour and strong heat flavour (Yokotsuka, 1985).

Table 2.1 Annual production of soy sauce marked and nonmarked by the Japanese Agricultural Standard (JAS) in 1982 in Japan.

JAS Grades	Kiloliters	Percent
Special[1]	674,093	56.9
Upper	289,911	24.5
Standard	106,176	9.0
Non-JAS	114,122	9.6
Total	1,184,302	100.0

[1] Special grade is granted for the soy sauce which consists of fermented soy sauce only.
Adapted from: Fukushima, 1985.

Table 2.2 Typical compositions of five varieties of soy sauce recognized by the Japanese government.

	Soy Sauce				
	Koikuchi-shoyu	Usukuchi-shoyu	Tamari-shoyu	Saishikomi-shoyu	Shiro shoyu
Baumé	22.0	22.2	29.9	26.9	26.9
NaCl (g/100 mL)	16.9	18.9	19.0	18.6	19.0
Total N (g/100 mL)	1.57	1.19	2.55	2.39	0.50
Formol N (g/100 mL)	0.94	0.80	1.05	1.11	0.24
Reducing Sugar (g/100 mL)	3.0	4.2	5.3	7.5	20.2
Alcohol (vol/100 mL)	2.3	2.1	0.1	Trace	Trace
pH	4.7	4.8	4.8	4.8	4.6
Colour	Deep brown	Light brown	Dark brown	Dark brown	Yellow to tan

Adapted from: Fukushima, 1985.

Usukuchi-shoyu, a light-coloured soy sauce, is made from a mixture containing more wheat and less soybeans than koikuchi type (Yokotsuku, 1985). Fukushima (1985) reported that the ratio of soybeans to wheat in this type of soy sauce is the same as in koikuchi-shoyu, except that fermentation is done under conditions that prevent the colour development. Saccharified rice-koji, with water, called Amasake, is sometimes added to usukuchi mash to ameliorate the salty taste. The final product, a lighter reddish-brown sauce, is of a milder flavour and aroma and is used mainly for cooking when one wishes to preserve the original colour and flavour of the foodstuff. Usukuchi-shoyu is primarily used in a western area of Japan.

Characterized by a slightly higher content of amino acids and lacking aroma, Tamari-shoyu is the third variety of soy sauce in Japan which is consumed locally around the Nagoya area. Only a small amount of wheat is used, sometimes none at all, but the main material of the koji is soybeans. Often, the ratio of materials making the koji is 10:1-2, of soybeans to wheat.

The next variety is Shiro-shoyu, a yellow to tan-coloured product which is made mainly from wheat and very little soybeans. Conditions for its fermentation prevent the development of the usual dark colour associated with koikuchi-shoyu and it has a low content of amino acids.

Saishikomi-shoyu is the last variety of soy sauce and is characterized by aroma and full-bodied taste. It is made by enzymatic degradation of a mixture of equal portions of soybeans and wheat to make the koji, and then mixed with raw soy sauce instead of the usual salt solution. As with Shiro-shoyu, it is produced and consumed only in isolated localities or for special industrial uses in Japan.

2.1.3 Production and trade

The Japanese manufacturers of soy sauce are assumed to be less than 3,000 in number (Yokotsuka, 1985). Five of the biggest manufacturers, namely Kikkoman, Yamasa, Higashimaru, Higeta and Marukin, account for about 50% of the total production. Table 2.3 shows the annual production of the five varieties of soy sauce. Yokotsuka (1985) reported

Table 2.3 Annual production of soy sauce marked by the Japanese Agricultural Standard (JAS) in 1982 in Japan.

Varieties	Annual Production (kL)	Percent
Koikuchi-shoyu	902,862	84.4
Usukuchi-shoyu	138,261	12.9
Tamari-shoyu	20,885	2.0
Shiro-shoyu	5,042	0.4
Saishikomi-shoyu	3,130	0.3
Total	1,070,180	100.0

Adapted from: Fukushima, 1985.

that the annual consumption of shoyu per capita in Japan is about 10 litres, of which 4.4 litres is consumed in the home and the remainder, 5.6 litres, institutionally and industrially.

In 1982, Wood presented a concise report on the trade in soy sauce. He stated that figures relating to exports suggest only a tiny part of the total soy sauce production in Japan, since soy sauce is widely made on domestic scale for private consumption. He concluded that the total trade in soy sauce is very substantial and is exhibiting steady growth in real terms, with the increasing European and American markets making a significant contribution to this growth. Recently, though, Koikuchi-shoyu has penetrated the U.S. market through supermarkets and the production of Kikkoman Foods, Inc. As much as 22,000 kL (kilolitres) per year was reported in 1983. In 1982 alone, an estimated 50,000 kL was the annual consumption, of which 20,000 kL was produced by Kikkoman and 21,000 kL by LaChoy and Chung King. Only 3,400-3,500 kL of soy sauce was imported from Japan, 3,200-3,300 kL from Hong Kong and 2,300 kL from other countries (Fukushima, 1985).

2.2 Manufacture

2.2.1 Introduction to manufacturing practises

As mentioned previously, soy sauce is divided into two groups, fermented soy sauce, which is the conventional one, and chemical soy sauce. The chemical sauce is not recognised nor defined as soy sauce but only as an extender of soy sauce in Japan. Its partly improved form, the semi-chemical type, is essentially a chemical soy sauce due to the content of undesirable compounds, especially in the form of organic acids. Figure 2.1 compares the organic acids found in fermented and chemical soy sauces. The undesirable compounds present in chemical soy sauce include dark humins, furfural, dimethyl sulphide, hydrogen sulphide, levulinic acid, formic acid and the like (Fukushima, 1985), which are not present in fermented sauce. Lactic acid is the main organic acid in fermented sauce, but formic acid is dominant in chemical sauce. The presence of levulinic acid, which does not exist naturally but which is found in chemical soy sauce, can determine if fermented soy sauce has been

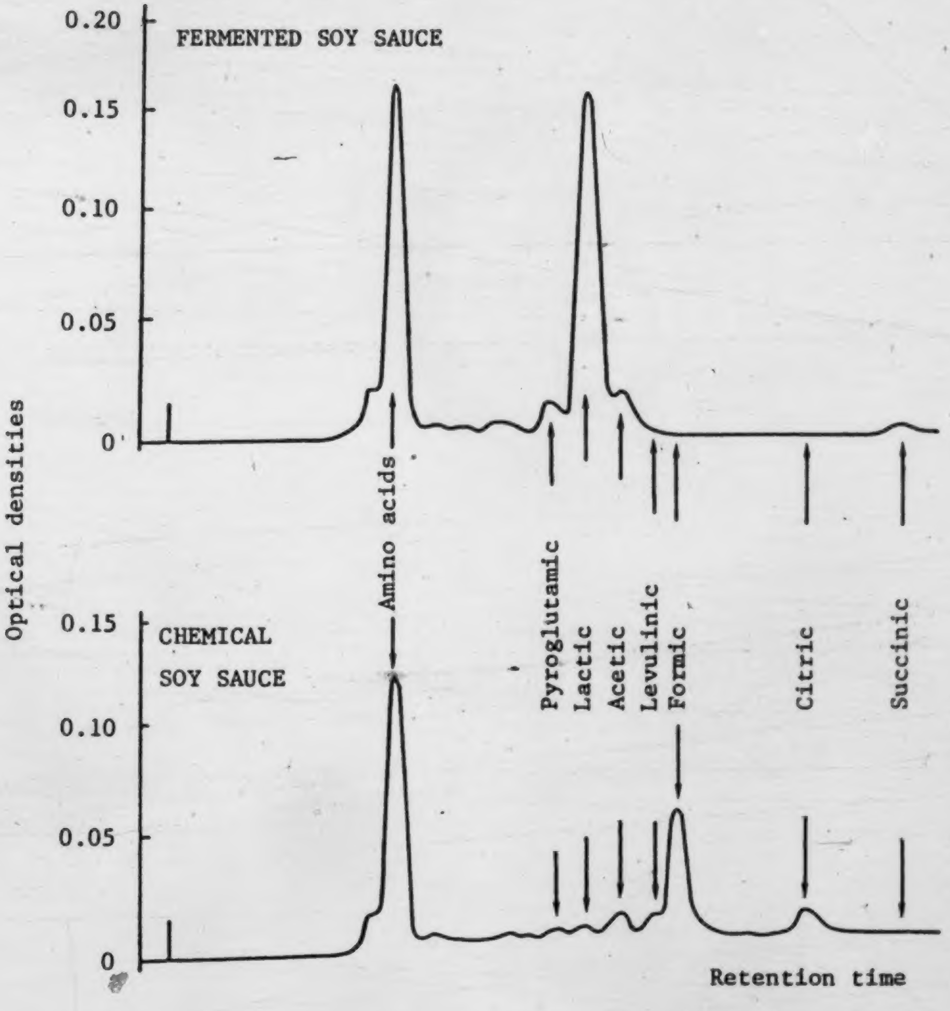

Figure 2.1 Chromatograms of organic acids in fermented and chemical soy sauce (adapted from: Fukushima, 1985).

adulterated with some chemical sauce.

2.2.2 Summary of Japanese Koikuchi-shoyu manufacture

Wood (1982), Fukushima (1985), Yokotsuka (1985) and several other workers have outlined the basic steps involved with the processing of soy sauce. Categorizing the steps into five main processes, Yokotsuka (1985) lists these steps as:

1. treatment of raw materials;
2. koji making process;
3. mash making and aging (Brine Fermentation Process; Fukushima, 1985);
4. pressing (Refining Process; Fukushima, 1985);
5. refining (Refining Process; Fukushima, 1985).

These steps are shown schematically in Figure 2.2.

2.2.2.1 Treatment of raw materials

Initially, whole soybeans or, more commonly, defatted soybean flakes or grits are moistened and cooked under pressure, thereby influencing the digestibility of soybean protein. Wheat, on the other hand, is roasted for a few minutes at a temperature between 160-180°C, then coarsely ground into 4 or 5 pieces (Fukushima, 1985; Yokotsuka, 1981, 1985).

2.2.2.2 Koji making process

The digested soybeans and ground wheat, mixed together in equal proportions, are inoculated with a pure cultured starter of ***Aspergillus oryzae*** or ***sojae***, called the "koji starter" or "seed mould". The mixture is spread on shallow perforated vats or stainless steel plates and then aerated for 3 days with a temperature of 30°C and moisture-controlled air. A decrease in moisture content of materials from about 40-45% to 25-35% allows growth of the mould with a yellowish-green appearance as a result of sporulation. This is koji and it contains the necessary enzymes to hydrolyse proteins and carbohydrates in the raw materials into sugars, peptides and amino acids.

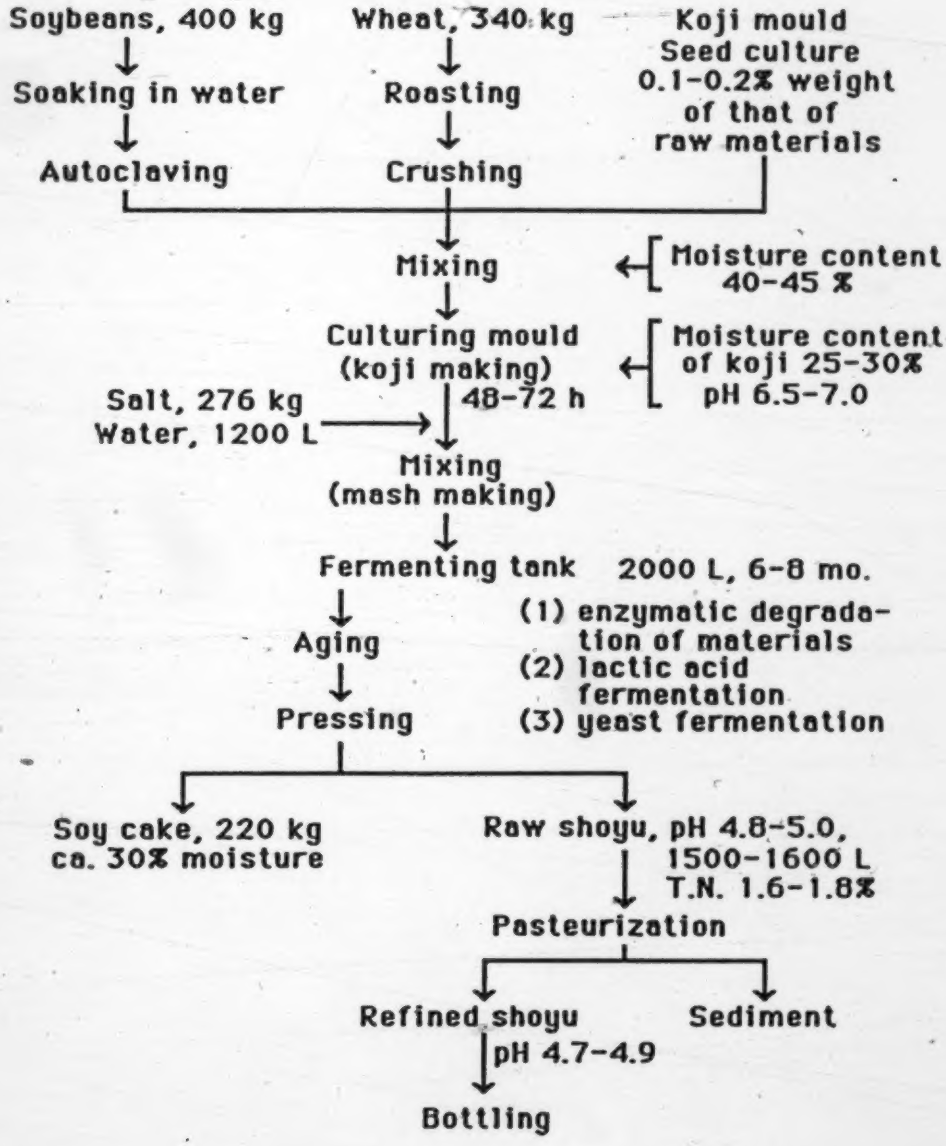

Figure 2.2 Koikuchi-shoyu fermentation. Adapted from: Yokotsuka, 1985.

2.2.2.3 Mash making and aging (brine fermentation)

Transferring the koji into fermentation vessels and then mixing with saline water of 22-25% (w/v) salt are the initial steps for the brine fermentation. A saline solution of about 120-130% volume of that of raw materials is used. This mash or "moromi mash" as it is called is held for 4-8 months, under appropriate temperature controls, with occasional aeration to mix the contents and to stimulate microbial growth. As fermentation proceeds, enzymes from koji hydrolyse most of the proteins to amino acids and low molecular weight peptides. Most of the starch is converted to simple sugars, which are fermented primarily to lactic acid, alcohol and carbon dioxide by lactic acid producing bacteria and yeasts. Only about 20% of the starch is consumed by the mould during koji cultivation (Yokotsuka, 1985). Most Japanese workers have reported the presence and use of ***Pediococcus halophilus*** (lactic acid bacteria) to produce lactic acid, which lowers the pH of the mash from an initial value of 6.5-7 to 4.7-4.9.

Other reports have indicated the production of lactic acid by ***Lactobacillus delbrueckii*** (Yong and Wood, 1974). The reduction in pH stimulates yeast growth, thus lactic acid fermentation is replaced by yeast fermentation, resulting in vigorous alcoholic fermentation. ***Torulopsis*** strains have been observed to grow at the middle and last stage of moromi fermentation, producing phenolic compounds and adding some aroma to the sauce (Fukushima, 1985). This is another group of salt-resistant yeasts.

Recent techniques have established a controlled microbial presence during the brine fermentation, whereby pure-cultured lactic-acid producing bacteria and yeasts are used to produce a constant desirable quality sauce.

The salt concentration effectively limits the growth of microorganisms to a few desirable osmophilic types and it stabilizes finally at a concentration of 17-18% (w/v).

2.2.2.4 Refining process

Fukushima (1985) put together the pressing of the mash and refining as the refining process. This includes filtering and pasteurization by heat. The aged moromi mash is wrapped in a cloth and pressed with hydraulic press until the moisture content of the residue is less than 25%. The filtrate, which is the raw soy sauce, is heated to 70-80°C by a plate heater and stored in a semi-closed tank where settlement of the coagulum results in a clear supernatant. This clear supernatant is bottled or canned.

2.3 Processing and Raw Materials

Readily available and cheap raw materials have been used as the ingredients for soy sauce production.

2.3.1 Soybeans

Yokotsuka (1960) observed that there were slight differences in the chemical composition of soybeans from different countries. This could probably be due to geographical conditions and may cause slight differences in composition of soy sauces produced.

Defatted soybean grits are usually used in the preparation of soy sauce. Yokotsuka (1985) reported that only 3.2% of the total soybeans used in the production of soy sauce in 1978 was whole soybeans. The choice of the defatted soybeans is based basically on cost, enzymatic digestibility of proteins, fermentation period, and relative difficulty in manufacturing, especially in koji making and mash controlling. Also important are the quality of the sauce, in terms of chemical components such as glycerol, alcohol and lactic acid, organoleptic evaluation, and the stability of the product (Yokotsuka, 1985).

However, Wood (1982) reported that members of the whole food and "macrobiotic" food trades preferred the use of intact beans in these markets, and that whole beans seemed to be widely used throughout southeast Asia.

2.3.2 Wheat

Wheat is the carbohydrate source for culturing the mould and in fermentation of the mash. Whereas Wood (1982) considers a low-protein wheat as suitable, Yokotsuka (1985) reports that high-protein wheat kernels are preferred. Studies by some Japanese authors suggest that aromatic compounds such as vanillin, vanillic acid and 4-ethylguaiacol, are produced on cooking wheat and these contribute significantly to the aroma and flavour of the sauce (Wood, 1982).

The role of wheat in soy sauce production, reported by Yong and Wood (1974) to have been listed by Yokotsuka in 1964, is as follows:

1. To make the moisture content of material to be cultured with mould just adequate for mould growth. It must be about 45% in order to minimize the damage due to the growth of undesirable bacteria. Cooked soybeans have about 60% moisture, so roasted and crushed wheat serves to decrease the moisture of the material.

2. To assist in obtaining the highest proteolytic activity from the koji -- the activity is highest when the starting material is an equal mixture of soybeans and wheat -- along with greater growth of the mould.

3. To serve as the major source of carbohydrates as the precursor of sugars, alcohol and organic acids.

4. To serve as the source of lignin and glycosides, the precursors of vanillic flavour of shoyu.

5. To serve as a rich source of glutamic acid, an important taste ingredient in shoyu.

2.3.3 Ratio of soybeans to wheat

The use of wheat decreases the nitrogen content of soy sauce but it contributes aroma, flavour and glutamic acid. The best soy sauce is believed to be made from a soybean-to-wheat ratio of 50:50 by weight (Yokotsuka, 1960; Yong and Wood, 1974), or 52:48 by volume (Yokotsuka, 1960). Any adjustment to the ratio of the materials would result in a sauce of different quality. Differences arise in moisture content as well as nitrogen and carbohydrate contents.

2.3.4 Salt

Aside from acting as a preservative, sodium chloride exerts a selective action on the microorganisms which grow during the fermentation of the sauce. As such, an exclusive development of flavour and aroma-forming yeasts and lactic acid bacteria results, as reported by Yong and Wood (1974) from studies by Yong in 1971. Salt eliminates all dangerous anaerobic bacteria, such as *C. botulinum*, which otherwise would thrive on the soybean-wheat mixture. Yong and Wood (1974) reported that the Japanese used sea salt, and also observed that commercial sodium chloride, though containing foreign substances other than basic salts of calcium and magnesium and other impurities, produces good sauce. There does not seem to be any interference from the impurities. Purified sodium chloride has been used successfully for laboratory work only occasionally in Japan. They also reported the successful use of a "laboratory reagent grade" sodium chloride, on a laboratory scale, but stated that the application of pure cultured yeasts and Lactobacilli may have influenced the fermentation results.

2.4 Biochemistry and Microbiology of Koji and Moromi

2.4.1 Koji

Sections 2.2.2.1 and 2.2.2.2 as well as most of section 2.3 introduced koji as a part in the process of producing soy sauce. Simply put, koji is just an enzyme preparation produced by growing a mould, such as *Aspergillus oryzae* or *A. sojae*, on steamed rice or other cereals, and grains.

Malt is used widely in the Western world as an enzyme source in the preparation of foods such as beer and whisky. Likewise, koji has been used widely for centuries in the Orient as an enzyme source. It is used in the preparation of soy sauce and miso, a fermented paste-like product from soybeans and cereals, as well as in the production of the traditional Japanese alcoholic beverage made from rice (Yong and Wood, 1974; Fukushima, 1985).

Basically, the uniqueness of koji lies in the utilization of its amylolytic and/or proteolytic enzymes to convert starch into fermentable sugars and to break down proteins and polypeptides into amino acids.

To obtain a good quality koji, Yokotsuka (1985) indicated that it is necessary:

1. to obtain sufficient growth of mycelia;
2. to produce maximum amounts of enzymes needed, such as protease, amylase, and other plant degrading enzymes;
3. not to destroy the activity of enzymes once produced;
4. to minimise the consumption of starch caused by the growth of mould;
5. to avoid bacterial and mould contamination.

2.4.1.1 Koji moulds

Strains of *Aspergillus* moulds have been used for centuries to produce soy sauce and other fermented products. The species employed include *A. oryzae* and *A. sojae*, both of which belong to the same group and are characterized by the absence of mycotoxins (Wood, 1982). Seed koji, produced by culturing *A. oryzae* or *A. sojae* on either steamed polished rice (usual practice in Japan) or a mixture of wheat bran and soybean flour (China) is the starter culture used to inoculate the soybean-wheat mixture to be fermented.

Yokotsuka (1985) listed certain points to be considered when choosing strains of *Aspergillus* for food fermentations. These points included:

1. flavour and colour of the final product;
2. good spore forming ability, which is necessary to prepare the seed starter;
3. strong and rapid growth;
4. high enzymatic activity, especially of proteolytic and macerating enzymes;
5. lower consumption of starch during growth;
6. genetic stability;
7. length of stalk (short stalk strains are most suitable for mechanical koji cultivation);
8. no production of toxin.

The "seed koji" to be used as mould starter, having been incubated at 25-30°C for 75-100 h, produces as many as 1-2 x 10^9 spores per gram (Yokotsuka, 1985) of the inoculated pure mould. Traditionally, the inoculated koji mixture was put into small wooden trays and kept for 3 to 4 days in the koji-making room. Although this practice is still followed in many factories, modern automatic koji-making processes have been developed to replace the traditional method. Aidoo *et al.* (1984) proposed mechanized methods for improving technology at the koji stage. The control of temperature and moisture during the growth of the mould is important and usually the culturing temperature is around 25-35°C. As the mould grows, the temperature of the material increases and could get as high as 40°C. Such a temperature inhibits mould growth, and several authors, including Yokotsuka (1960, 1985), Fukushima (1985), Yong and Wood (1974, 1976) and Wood and Yong (1974), have stressed the importance of cooling the koji, by stirring. Cooling is usually done two times, at about 20 and 40 hours after inoculation (Yong and Wood, 1974, 1976).

The mature koji, after 72 hours of incubation at 30°C, has a clear yellow to yellowish-green colour on the surface and throughout the whole mass (Yong and Wood, 1974). Whereas 0.1-0.2% of "seed koji" or starter mould added to the mixture of wheat and soybeans has been the usual practice in making koji (Yokotsuka, 1960, 1985), studies by Wood and Yong (1974, 1976) have employed suspensions of washed fungal spores as inoculum.

Lotong (1985) reports the finding by Bhumiratana *et al.* (1980) that *A. flavus* var. *columnaris* has been isolated from Thai soy sauce koji, and has been shown to produce improved quality soy sauce when used as inoculum alone. It was also shown that it did not produce aflatoxin.

Koji enzymes

Koji has been described as a unique source of enzymes. The production of the enzymes involves a unique technology, whereby microorganisms are cultured on a solid medium of cereals (Fukushima, 1985). This technology has been described thoroughly in recent years (Hesseltine, 1972, 1977; Cannel and Moo-Young, 1980; Aido *et al.*, 1982; Steinkraus, 1984) as the Solid State (Solid-Substrate) Fermentation.

Koji, prepared in this manner, contains macerating, amylolytic and proteolytic enzymes in large quantities and in a well-balanced ratio (Fukushima, 1985). The proteolytic enzymes have an unique composite system of proteinases and peptidases. Together, the system breaks down the cell structure, hydrolysing most of the cell components, into smaller units. The most unique features of koji, as enzyme source, are that:

1. koji is the solid culture of green-yellow *Aspergilli*.
2. the proteins can be hydrolysed to the extent of almost free amino acids, with a high yield (Fukushima, 1985).

The macerating, amylolytic and proteolytic enzymes reported to be present in soybean koji include various classes of enzymes, namely sucrase, proteinases, lipase(s), phosphatase(s), cellulase(s), amylases and maltase (Wood, 1982). Among these, proteolytic enzymes hydrolysing the raw materials have been considered the most important reaction for the production of soy sauce (Nakadai *et al.*, 1973). Fukushima (1985) reported that 90-92% of the proteins contained in the raw materials for soy sauce production are hydrolysed into the liquid phase as free amino acids by proteolytic enzymes.

Several research papers, including those by Yong and Wood (1974, 1976, 1977a,b) and Geol and Wood (1978), report on the sequence of enzyme release and the hydrolytic products thereof.

Proteolytic enzymes

Also referred to as proteases, these enzymes contribute immensely to the final quality of soy sauce by hydrolysing crude proteins into peptides and amino acids. As such, the product yield in terms of soluble nitrogen is increased, giving soy sauce its palate fullness and nutritional value.

The proteinase complex is divided in three. These three divisions are acid, neutral and alkaline proteinases, which are also subdivided. Table 2.4, from Fukushima (1985), shows the proteinases found in koji. The proteinases degrade proteins into peptides and not individual amino acids.

Fukushima (1985) gives an account of the proteolytic enzymes in soy sauce koji. Other workers have also investigated the presence and activities of these enzymes and have

Table 2.4 Proteinases in koji.

Proteinase	MW x 10^3	Optimum pH	Activity[1]	Enzyme Weight[2]
Alkaline	23	10.5	929	418
Semialkaline	32	8.3	55	---
Neutral I	41	7.0	80	131
Neutral II	19	6.0	9	152
Acid I	39	3.2	44	617
Acid II	100	3.0	10	---
Acid III	31	3.0	5	---

[1] Unit against casein per gram of koji.
[2] Microgram per gram of koji.
Adapted from: Fukushima, 1985.

reported on them (Yong and Wood, 1977a; Impoolsub *et al.*, 1981; Nakadai *et al.*, 1973a-g).

Table 2.5, after Yokotsuka (1985), lists the kinds of enzymes that have been isolated from koji mould.

The three types of fungal proteinases, characterized by pH, exhibit distinct specificities (Wood, 1982), and are all endo-type proteinases. These have been categorized such that neutral proteinases or proteases are said to be specific for hydrophobic amino acid residues, on the amino side of the bond being broken. Kundu and Manna (1975) reported the formation of both neutral and alkaline proteases in *A. oryzae*, while Sekine (1976) reported on two types of neutral proteases in *A. sojae*. Unlike neutral proteases, alkaline proteases are specific for aromatic or hydrophobic amino acid residues located at the carboxyl side of the bond undergoing hydrolysis. The pH optimum is around 10 (Wood, 1982). On the other hand, the acid proteases with pH around 3 to 4 are said to be specific for aromatic or hydrophobic amino acid residues at both sides of the bond being hydrolysed.

Initially, koji is at a neutral pH and, as such, the mould enzymes which predominate would be the neutral and alkaline proteases (Lotong, 1985; Impoolsup *et al.*, 1981; Yong and Wood, 1974). The early moromi stage will also have these two enzymes dominating the proteolytic enzymes while the activity of acid protease is minimal. In the presence of 18% salt, the activities of neutral and alkaline proteases decrease considerably and, at the reduced pH of moromi, the acid proteases are thought to be the chief source of proteolysis (Wood, 1982).

Peptidases, present in koji as exo-type peptidases, liberate amino acids from the carboxy- or amino-terminal of peptides successively (Fukishima, 1985). Usually present as carboxypeptidases and aminopeptidases, they have been found to be specific in their reactions, especially since all four carboxypeptidases have optimum pH in the acid region, while the seven aminopeptidases are specific for the amino-terminal leucine. They are commonly referred to as acid carboxypeptidases and leucine aminopeptidases. Altogether, they liberate amino acids, including glutamic acid and glutamines. Part of the glutamines is converted to glutamic acid by the action of glutiminase of the koji mould, and the rest is converted to pyroglutamic acids. The importance of glutaminase, as part of the enzymatic digestion taking

Table 2.5 Enzymes isolated from koji mould.

	Molecular weight x 10^3	Isoelectric point
Leucine-aminopeptidase	26.5	3.3
	40	3.9
	61	4.1
	99	4.4
	145	6.1
Acid carboxypeptidase	43	2.1
	61	4.2
	125	4.4
		6.0
Acid proteinase	36	3.4
	55	4.1
	120	4.6
Neutral proteinase I	45	4.3
Neutral proteinase II	19	5.8
Alkaline proteinase	22	7.8
Semi-alkaline proteinase	32	6.5
α-amylase	23	3.6
Glucoamylase	80	5.8
Carboxy methyl cellulase	17.5	3.6
	22	4.3
	31	8.5
	89	9.6
Glutaminase	81	3.9
		4.6

Adapted from: Yokotsuka, 1985.

place in koji, is observed in the increase of glutamic acids, the most important flavour component of soy sauce. Figure 2.3, from Fukushima (1985), sums up the role of each enzyme in koji during protein hydrolysis.

Yong and Wood (1977a), assayed for protease activity in a bid to detect the overall protein hydrolysis in an experimental koji. Protease activity was detected from the start, even though the level was very low, and despite the fact that *Aspergillus* spores had been washed three times to prevent carry-over of enzymes from the old mycelium (Yong and Wood, 1976). A similar observation was made by Impoolsup *et al.* (1981). Figure 2.4, adapted from Yong and Wood (1977a), shows the protease activity in koji made from *A. oryzae* strain 1989. (See Appendix for definition of enzyme activities.) From the graph (Figure 2.4), protease activity is seen to increase rapidly between the 20th and 30th h of incubation, reaching a maximum around the 40th to 50th h. Thereafter, protease activity declined. Impoolsup *et al.* (1981) observed that, during the first 20 h, when activity of protease was low, the fungus growth was rapid. The rapid increment in protease activity coincided with the late growth and formation of spores.

Proteinase or protease activity was observed to decline sharply within the first two days of mixing koji with salt, as shown in Figure 2.5 (Yong and Wood, 1977b). Similar effects were observed by other workers. A plausible explanation for this behaviour is given by Yong and Wood (1977b), that an initial denaturation or precipitation of the enzyme occurs, followed by re-solubilization and/or the release of fresh enzyme upon lysis of mould hyphae. This could explain why an equally rapid increment in proteinase activity occurs soon after the initial decline.

Carbohydrate-hydrolysing enzymes

The enzymes in this category which have been reported include sucrase, endo-amylase or α-amylase (Yong and Wood, 1974, 1976, 1977a,b), cellulase and exo-amylase (Goel and Wood, 1978), amyloglucosidase and maltase (Aidoo *et al.*, 1981).

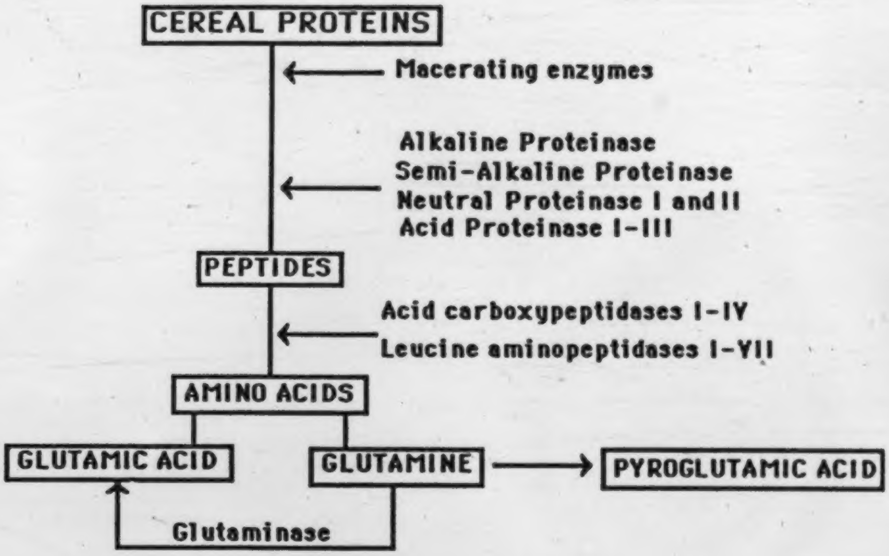

Figure 2.3 Role of each enzyme in koji during protein hydrolysis. Adapted from: Fukushima, 1985.

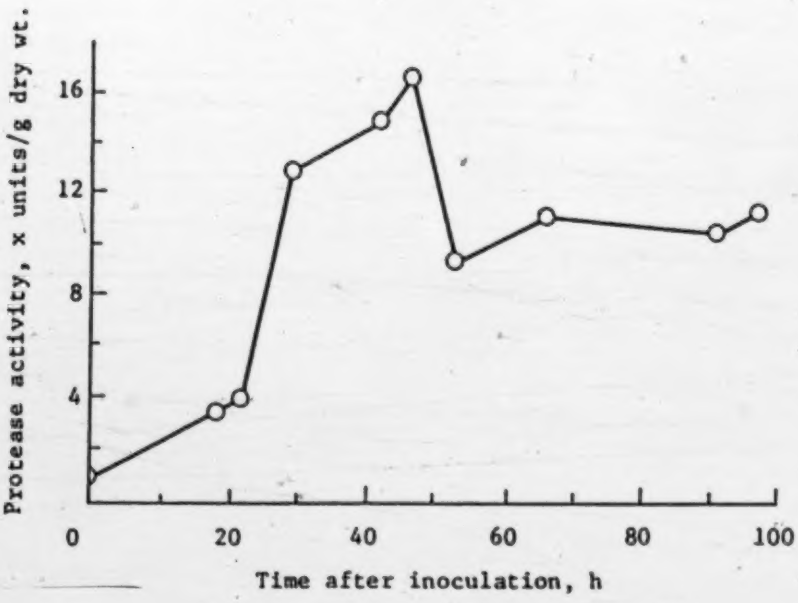

Figure 2.4 Protease in koji inoculated with *A. oryzae* strain 1989 (adapted from: Yong and Wood, 1977a).

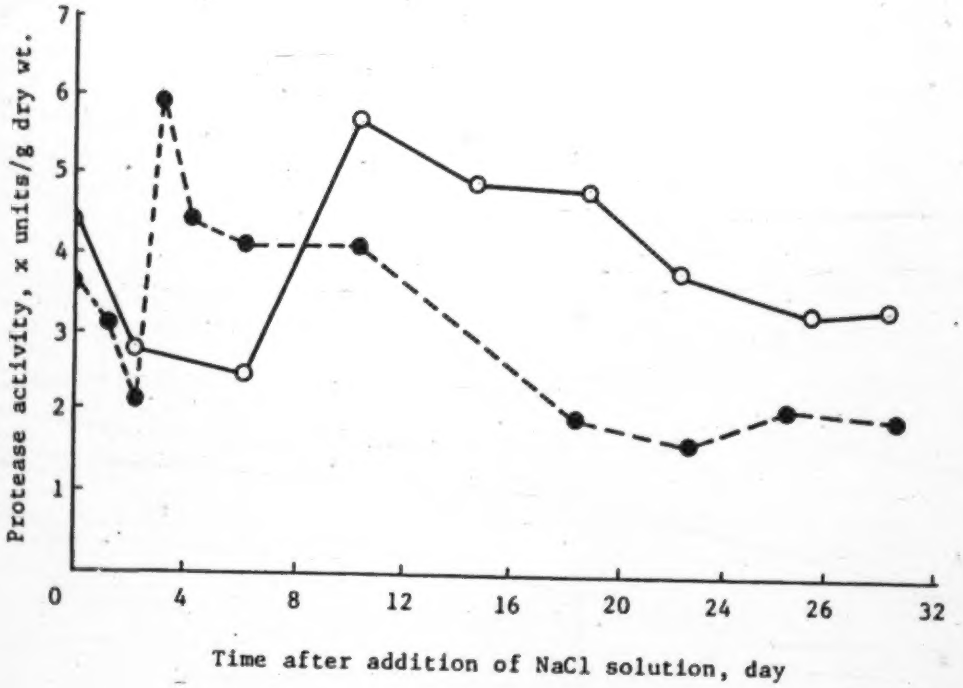

Figure 2.5 Protease in moromi (adapted from: Yong and Wood, 1977b).
(○) moromi with no microorganisms added.
(●) moromi with added *L. delbrueckii* and *S. rouxii*.

α-Amylase activity was detected after 20 h of koji incubation but, since reducing sugar level had increased during the first 20 h, Yong and Wood (1975, 1977a) were able to demonstrate the presence of sucrase and, consequently, attributed the production of reducing sugar to it. In the presence of 18% salt, there was an initial decline in α-amylase activity followed by a recovery (Yong and Wood, 1977b), as was the case for proteinases, but in general α-amylase was observed to be less stable, and the extent of loss of activity was very pronounced (Figure 2.6).

Goel and Wood (1978) did not differentiate between amyloglucosidase and β-amylase activities and reported on total activity in terms of reducing power, thereby incorporating the production of reducing end groups through α-amylase (endo-amylase) activity. The levels of exo-amylase in koji (Goel and Wood, 1978) increased more rapidly than endo-amylase levels (Yong and Wood, 1977a) after spore germination. This was followed by a period of slow rising of exo-amylase concentration between 30 h and 50 h of fermentation, and then a steady decline in activity during the remainder of the fermentation. This steady decrease differed from the behaviour of endo-amylase, which showed a fairly steady level during much of the koji fermentation, and a further increase towards the end. In the moromi stage, a marked contrast was observed in the stability of both amylases. It turned out that endo-amylase was less stable in moromi. Figure 2.7 shows the activities of exo-amylase in both koji and moromi.

The presence of cellulose-degrading enzymes assists in increasing yields of solubilized carbohydrates and proteins from the soybeans and wheat flour by disintegrating the cell walls. Goel and Wood (1978) measured cellulase activity such that the production of reducing power measured the overall cellulose degradation. Figures 2.8 and 2.9 show the activities of cellulase in both koji and moromi.

Aidoo *et al.* (1981) showed the activities of both amyloglucosidase and maltase (α-glucosidase) in koji (Figure 2.10). Despite the difficulty in distinguishing between the activities of these two enzymes (since amyloglucosidase has maltose-type activity), there is evidence that *A. oryzae* produces extracellular amylolytic enzymes, having both

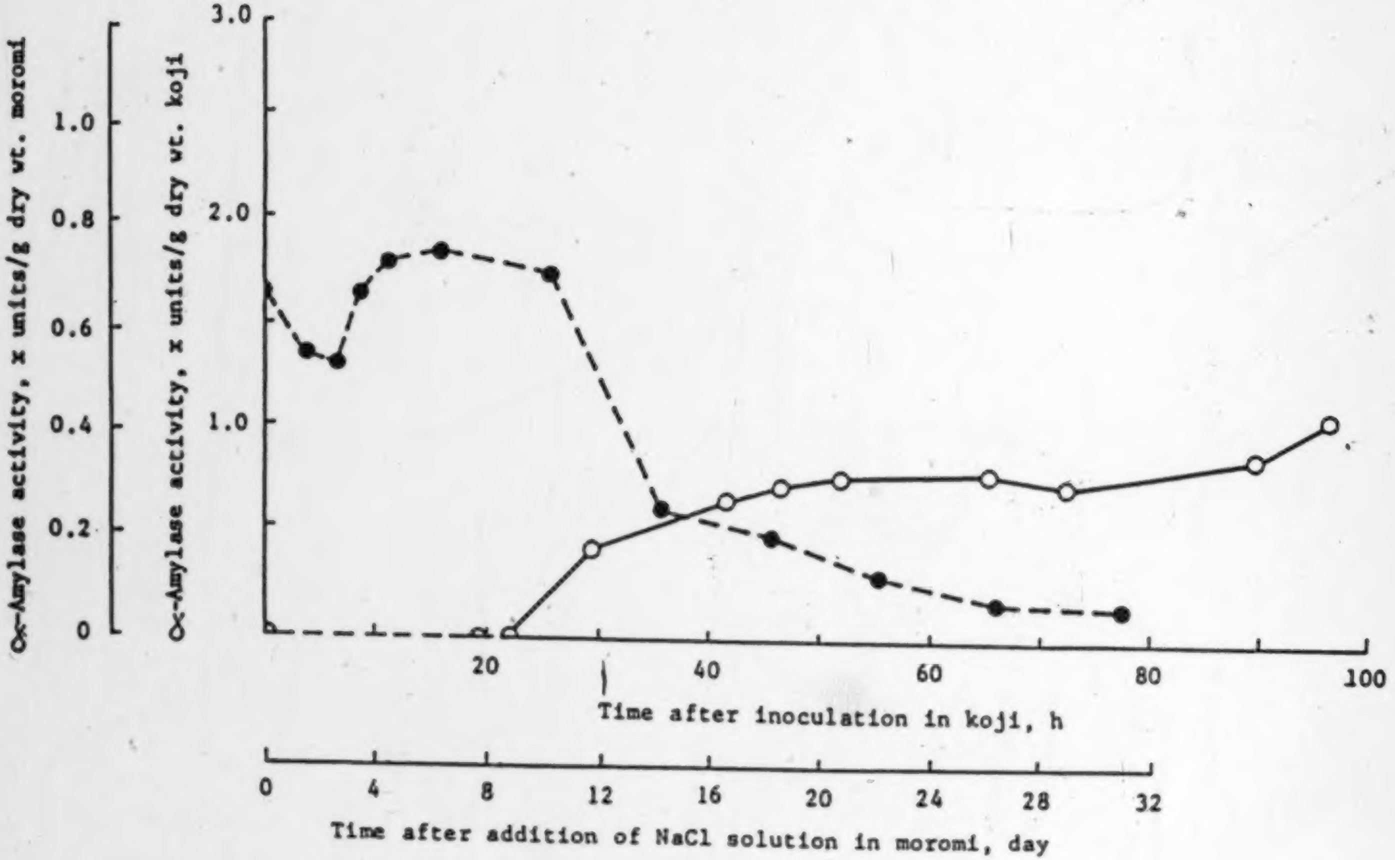

Figure 2.6 Amylase in koji (○) and moromi (●) (adapted from: Yong and Wood, 1977a,b).

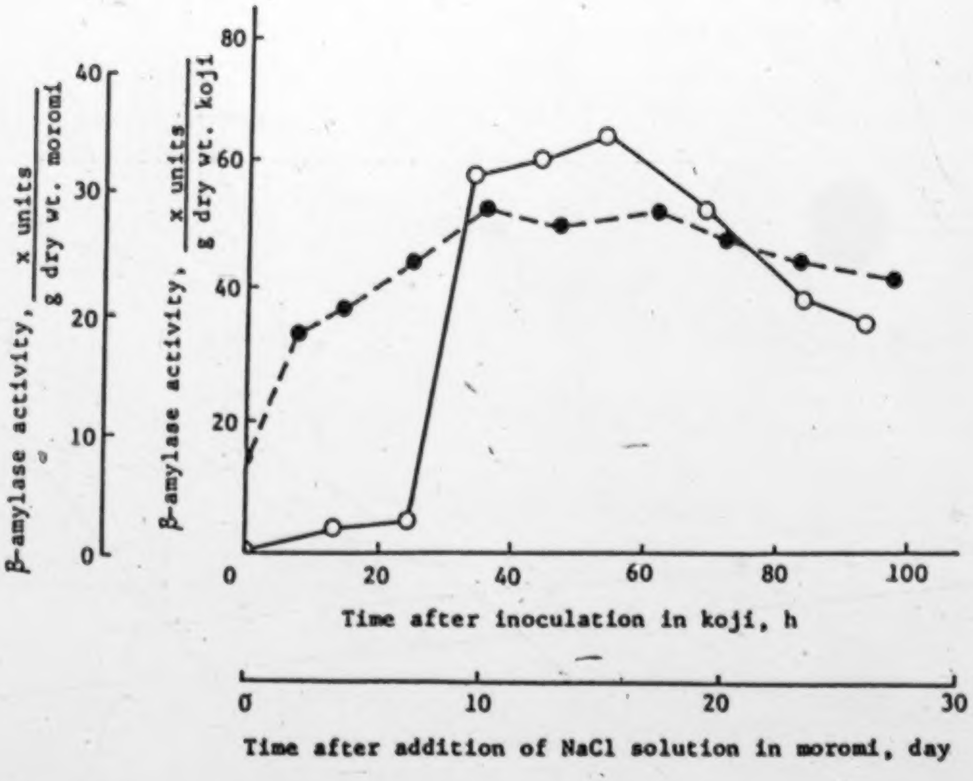

Figure 2.7 β-Amylase in koji (○) and moromi (●) (adapted from: Goel and Wood, 1978).

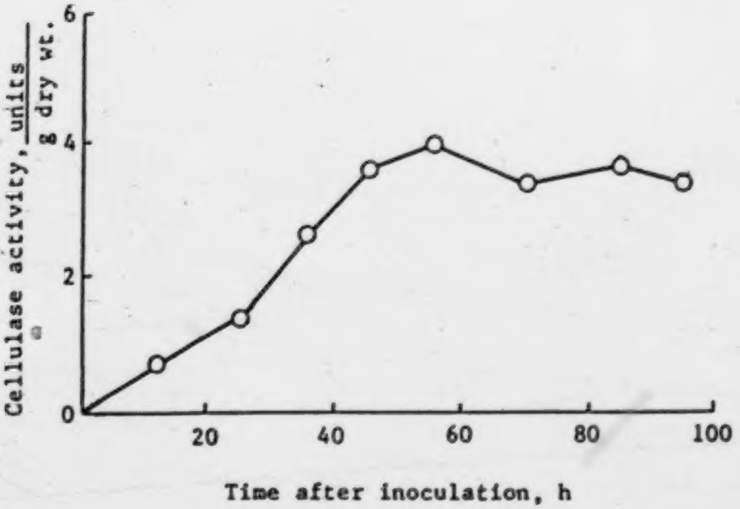

Figure 2.8 Cellulase in soy sauce koji (adapted from: Goel and Wood, 1978).

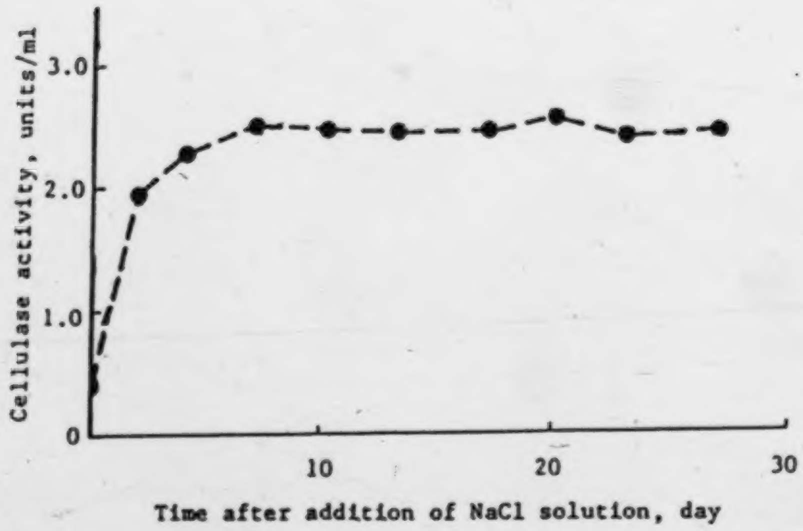

Figure 2.9 Cellulase in soy sauce moromi (adapted from: Goel and Wood, 1978).

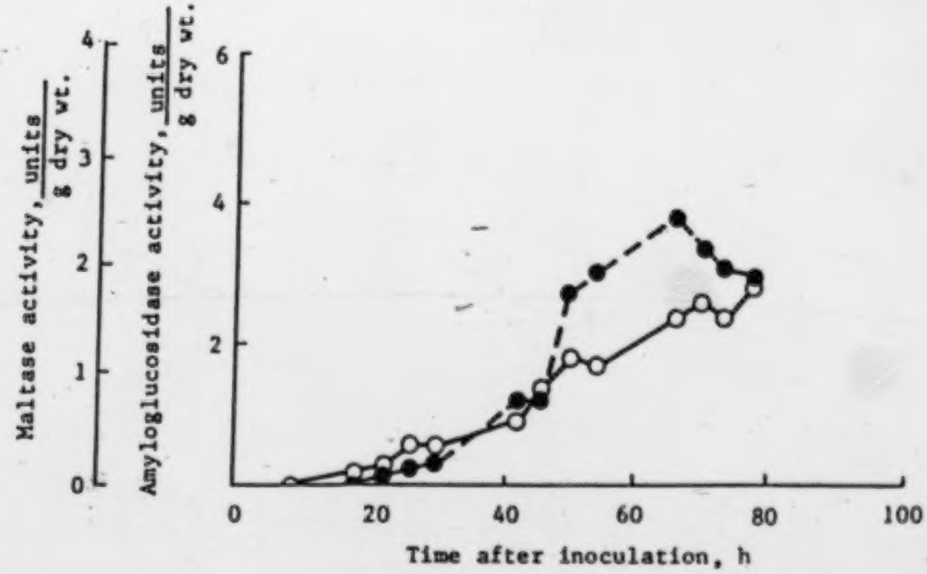

Figure 2.10 Amyloglucosidase (●) and maltase (○) activities in koji fermentation. (adapted from: Aidoo *et al.*, 1981).

amyloglucosidase and maltase activities, in a complex koji fermentation. Their report showed that maltase increased in concentration throughout the fermentation, resulting in the disappearance of maltose from the koji and the accumulation of glucose. Though amyloglucosidase was detected about 2 h after the appearance of maltase, i.e. at 20 h, its concentration decreased later on. Only small amounts of both enzymes were detected in the final soy sauce.

The production of the enzyme α-galactosidase has been reported by Smiley *et al.* (1976). Yokotsuka (1985) also cited a report by Ishii *et al.* (1972) on the production of some enzymes by *A. sojae* having a strong plant tissue degrading activity, which was attributed to pectinases. Pentosan-degrading enzymes, hemicellulase and β-galactosidase are some other enzymes which have been said to be involved in the enzymatic degradation of plant tissues of soybeans and wheat kernels in soy sauce fermentation. Altogether, they relate to the yield of sauce, the ease of pressing of the moromi mash, and the quality of the final product.

Other enzymes

Lipase, tyrosinase, phosphatases, deaminases, and nucleases are a few of the other enzymes thought to be present in soy koji.

Yong and Wood (1977a) reported on both lipase and tyrosinase. The presence of lipase causes the breakdown of lipids present in soybean into free fatty acids and some glycerol. Though glycerol contributes to the flavour of the sauce, the free fatty acids are undesirable in taste and have the potential of developing rancidity. The use of defatted soybean meal minimizes the level of free fatty acids.

Although Yong and Wood (1977a) did not detect tyrosinase in their soy sauce fermentation, Oba *et al.* (1974) detected the presence of tyrosinase in rice koji, and observed that the tyrosinase level correlated with the extent of browning in koji.

Wood (1982) mentioned the report by Aidoo (1979), suggesting the presence of extracellular deaminases in the last stages of koji fermentation. Pectinase was not detected by Wood and colleagues, though several papers report on its production. Kuninaka *et al.* (1980) have also reported the production of phosphatases, deaminases and ribosidases.

2.4.1.2 Chemical changes occurring in koji

Besides influencing the degree and speed of enzymatic degradation of raw materials in the salty mash, the quality of koji also influences the chemical and organoleptic quality of the final product (Yokotsuka, 1985). Wood and Yong (1974), reviewing changes occurring in koji development, cited the study done by Yong (1971). Table 2.6 shows these changes.

The presence of sucrase and α-amylase (Yong and Wood, 1977a) and β-amylase (exo-amylase; Goel and Wood, 1978) contributes to the production of reducing sugar throughout the koji fermentation. As indicated in section 2.4.1.1, the different periods associated with the production and release of these enzymes correlate with the reducing sugar levels (Figure 2.11). The total soluble nitrogen level increased steadily between 20-70 h after inoculation, corresponding well with the proteinase level in koji, while the amino nitrogen level fluctuated throughout (Figure 2.12). The amount of amino nitrogen present in koji suggests the extent of protein hydrolysis to amino acids and small peptides. Free ammonia is produced during koji fermentation, but the increase is very rapid after 40-50 h, coinciding with the onset of sporulation (Wood and Yong, 1974; Yong and Wood, 1977a). High levels of ammonia in soy sauce make it unacceptable, so together with other important parameters, including maximum amount of compounds and enzymes, koji is allowed to ferment for 72 h, after which it is terminated. Prolonged koji fermentation will result in a sauce having a mouldy off-flavour and excessive ammonia content (Yong and Wood, 1977a).

2.4.2 Moromi mash fermentation

Moromi mash fermentation, also called brine fermentation, was dealt with briefly in section 2.2.2.3. This is the second stage of fermentation, which is essential in soy sauce production so that a complex interaction of microorganisms under severe conditions in the soy mash will produce a subtly aromatic brew.

Long fermentation periods have been the tradition in sauce brewing, resulting in very good quality soy sauce. In addition, tradition requires the exposure of the mash being fermented to seasonal cycles of temperature. Today, though, manufacturers stress natural and

Table 2.6 Changes in composition of koji during soya sauce fermentation.

Time (h)	pH	Temp. (°C)	Moisture (% w/w)	Reducing sugars as glucose (g % dry wt)	Total soluble nitrogen (g % dry wt)	Amino nitrogen (g % dry wt)	Ammonia nitrogen (g % dry wt)
0	6.55	28.5	47.0	0.3	0.57	0	0.02
18	6.49	28.5	49.0	2.2	0.63	0.02	0.04
22	6.44	30.5	48.0	2.8	0.62	0.09	0.03
29.5	6.48	41.0	49.0	3.7	1.04	0.34	0.07
42.0	6.74	----	47.0	2.5	1.07	0.49	0.11
47.0	6.86	39.0	42.0	2.4	1.25	0.29	0.20
52.5	6.90	36.0	39.0	1.7	1.30	0.26	0.30
66.0	7.08	32.0	36.0	2.1	1.44	0.39	0.34
73.0	7.34	31.5	34.0	2.5	1.59	0.32	0.40
90.5	7.48	----	35.0	2.1	1.59	0.33	0.39
96.5	7.50	30.0	34.0	1.7	1.58	0.33	0.39

Adapted from: Wood and Yong, 1974.

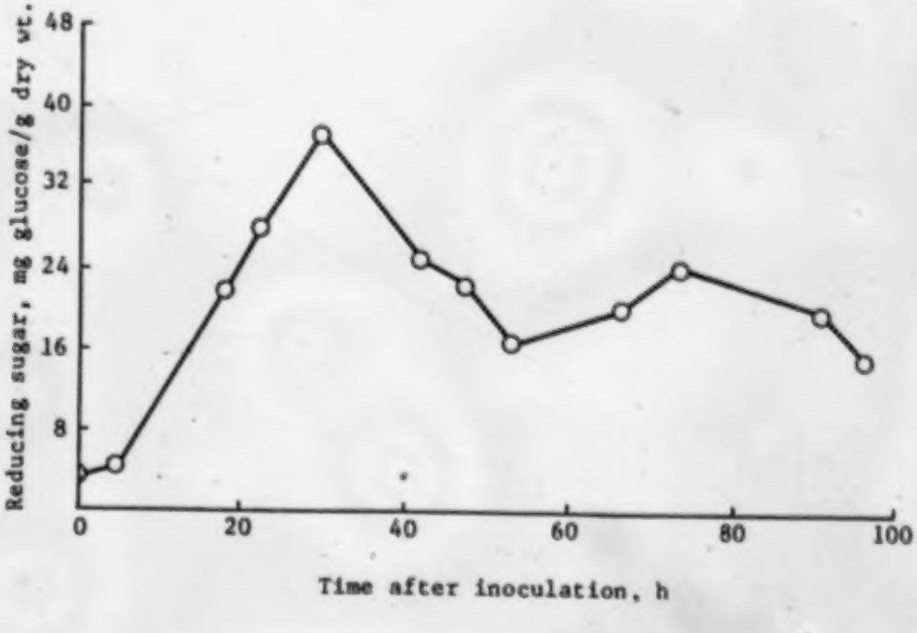

Figure 2.11 Reducing sugars in koji inoculated with *A. oryzae* strain 1989 (adapted from: Yong and Wood, 1977a).

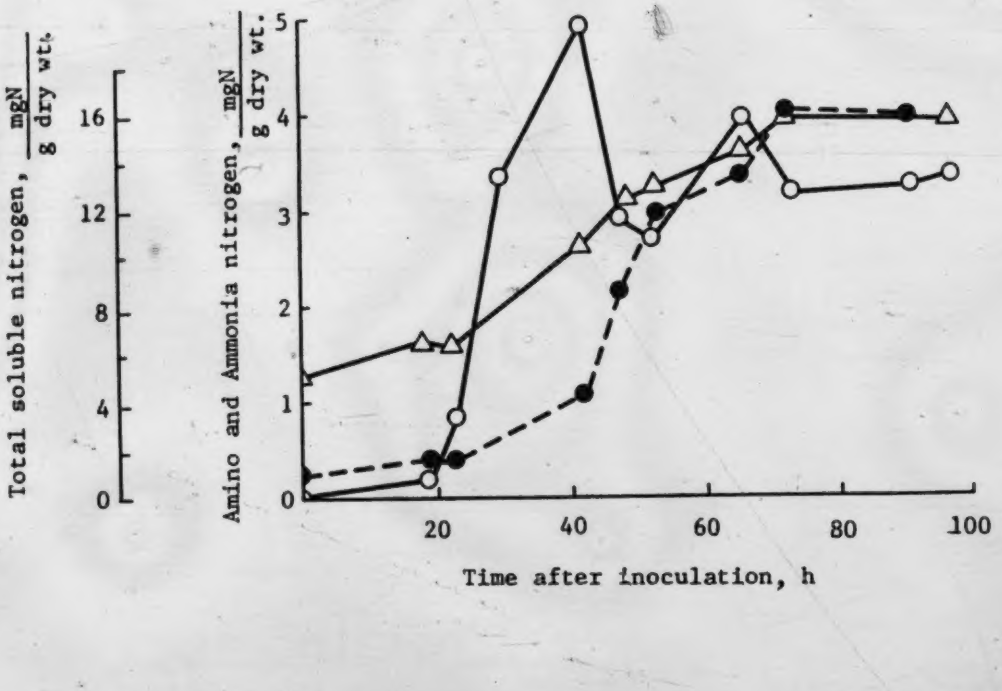

Figure 2.12 Total soluble nitrogen (△), amino nitrogen (○) and ammonia nitrogen (●) in koji inoculated with *A. oryzae* strain 1989 (adapted from: Yong and Wood, 1977a).

traditional forms of the process, good quality sauces have been produced in much shorter periods, and without the exposure to seasonal cycles of temperature.

2.4.2.1 Microorganisms during brine fermentation

Yokotsuka (1960) reported that sometimes other fungi, such as *Monilia*, *Penicillium* and *Rhizopus*, are present in mash, in addition to *Aspergillus oryzae* or *A. sojae*, but had no relation to proper aging. Fukushima (1985) mentioned several microorganisms as contaminants present in koji, such as some species of yeast, the genuses *Micrococcus*, *Streptococcus*, *Lactobacillus* and *Bacillus*, and attributed their presence to the non-aseptic operation of koji making. However, these contaminating microorganisms are not resistant to the high concentration of salt and therefore die within one or two months (Fukushima, 1985). It has been reported (Yong and Wood, 1976) that mould from the koji exists as spores in the moromi mash and throughout the fermentation, without germinating. The same is true of *Bacillus*, surviving as spores, but these spores are killed by high-temperature-short time pasteurization before bottling (Fukushima, 1985).

As a result of the composition of moromi, especially the high salt content of about 18% (w/v) sodium chloride, growth of microorganisms is limited to special lactic acid bacteria and yeasts which are resistant to high salt concentrations. Most Japanese researchers have reported the presence of *Pediococcus halophilus* as the sole bacterium vigorously growing in soy mash. Studies by Ho *et al.* (1984) confirmed the presence of *P. halophilus* in the brine phase of soy sauce fermentation, in Malaysia. Also, Japanese workers have reported the presence of *Lactobacillus* in the moromi, but not much significance has been attached to this. Lockwood (1947) claimed to have produced a good soy sauce using *L. delbrueckii* as the only bacterium in the fermentation. Following these reports, Yong and Wood (1976) used *L. delbrueckii* in their experimental soy sauce production and the quality of the sauce was found to be good.

The soy yeasts were found to be salt-tolerant *Saccharomyces rouxii*, which also tolerated high osmotic pressures. Yong and Wood (1974) reported the observations by Yokotsuka *et al.* (1967a) that two strains of yeasts were involved in the good flavour production and actual fermentation of soy mash. This and other reports indicate that the yeast strains were *S. rouxii* and *Torulopsis* species (halophilic yeasts). Yong and Wood (1976) produced a good sauce without *Torulopsis* species, whereas the Japanese reports involve *Torulopsis* to produce the characteristic flavour of aged mash.

The change in microflora during the brine fermentation is dependent on the conditions in the moromi mash. At an initial pH of about 6.5-7.0, with a salt content over 18% (w/v) and containing reducing sugars as well as many nutrients such as amino acids, base of nucleic acids and vitamins produced by the koji digestion, growth of *P. halophilus* is favoured. Growth of *P. halophilus* rapidly decreases the pH of the moromi mash to about 5 or below by the production of organic acids, mostly lactic acid. At this pH value, the lactic acid fermentation is taken over by *S. rouxii*. Yong and Wood (1976) reported that yeast will only grow up vigorously in soy mash when pH has dropped to below 5.5. They also cited Onishi's report of 1957 that "soy-yeasts" could only grow when pH lay between 4 and 5. *S. rouxii* cannot grow in 18% (w/v) salt when pH is above 6. Growth of *S. rouxii* is principally associated with the production of alcohol, after which the growth gradually decreases. Although *Torulopsis* species start growing at an early stage, the rate is slower than that of *S. rouxii*, which is more anaerobic than *Torulopsis* species (Fukushima, 1985). However, *Torulopsis* grows during the latter part of brine fermentation, taking over growth from *S. rouxii* and producing alkyl phenols and aromatic alcohols, as cited in reviews by Yong and Wood (1974) and Fukushima (1985). The higher accumulation of total nitrogen in the latter period of brine fermentation, and the presence of alkyl phenols and aromatic alcohols offer resistance to growth of *S. rouxii* such that fermentation is taken over by *Torulopsis* species.

The addition of pure culture microorganisms to moromi mash has been observed to accelerate the fermentation process and also to shorten the period required to produce the sauce.

Yong and Wood (1976) inoculated their mash with pure culture of *Lactobacillus delbrueckii* and *S. rouxii* and produced a good quality sauce in 31 days. Yokotsuka (1986) also reported that some investigators obtained good results when they added pure cultured

Lactobacilli to their new mash. He cited work done by Jose *et al.* (1976) in which an initial inoculum of 10^2-10^3 Lactobacilli in shoyu mash, during lactic fermentation, reached 10^8 after 3 months. A large inoculum, on the other hand, was found to decrease the pH value as well as decrease the protein digestibility.

Yokotsuka (1986) again cited other reports where pure cultured yeasts, *S. rouxii* and *Torulopsis* species were added to the mash. This accelerated the alcoholic fermentation and shortened its development time. In addition, the good volatile flavours were produced in the final product.

Properties of lactic acid producing bacteria

P. halophilus is a gram-positive micrococcus with a cell diameter of 0.6-0.9 μm (Ho *et al.*, 1984; Fukushima, 1985). It is halophilic and facultatively anaerobic. Fukushima (1985) reported that the optimum water activities for this bacterium are 0.99-0.94 with the lowest at 0.808, corresponding to 5-10% (w/v) and 24% (w/v) salt contents, respectively. In this high salinity, the growth temperature is elevated to about 42°C, though the lowest temperature in salt-free medium is around 20°C. *P. halophilus* grows well in the pH range between 5.5 and 9.0, and its optimum temperature for growth is 25-30°C.

On the other hand, Yong and Wood (1974) reported that some lactobacilli grow at temperatures up to 47°C, in more normal media. In their experimental soy sauce production in 1976, Yong and Wood employed a temperature of 40°C during the brine fermentation. They observed that growth of *L. delbrueckii* enhanced the rapid decrease in pH, by the continued production of lactic acid, leading finally to a decrease in viable numbers by the end of the fermentation period (Figure 2.13). The decrease in pH stimulates yeast fermentation, however indications by Wood (1982) and Wood and Hodge (1985) show that the lowering of pH is not the only role of the lactic acid bacteria. This opinion resulted from a difference in organoleptic assessment of soy sauce which had been produced by inoculation of pure cultured yeast on soy mash previously soured with lactic acid to a pH of 4.5 (Yong and Wood, 1976). Furthermore, Noda *et al.* (1980) recognised that *P. halophilus* (lactic acid bacteria) produced

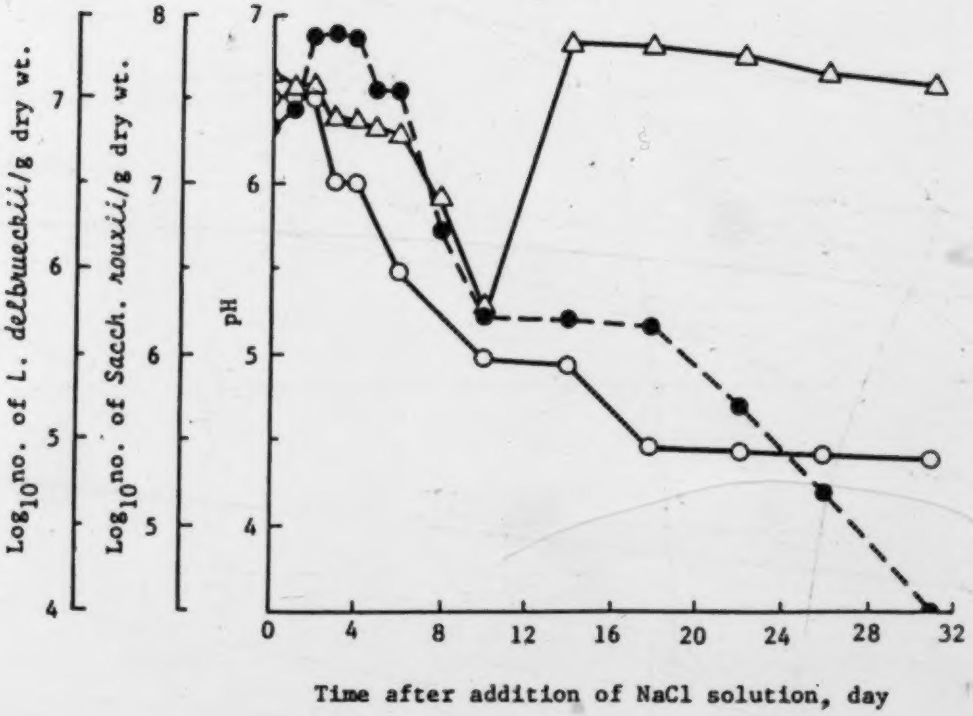

Figure 2.13 Viable counts of *L. delbrueckii* (●), *S. rouxii* (△) and pH (○) in soy mash (adapted from: Yong and Wood, 1976).

a metabolite, acetic acid, during the brine fermentation, and this had an inhibitory effect on the growth of osmophilic yeasts. They also reported that the lactic acid produced was slightly inhibitory. In 1982 a further investigation by Noda *et al.* revealed that this inhibitory activity of acetic acid was greatly increased as the pH of soy mash decreased.

Properties of yeasts

In soy sauce production, the principal alcoholic fermentation during the brine fermentation is the responsibility of the osmophilic yeast, *S. rouxii*. A salt-tolerant yeast, it can grow in a medium with as high a salt content as 24-26% (w/v), but it can also grow in salt-free media. The water activities corresponding to the high salt contents are in the range of 0.787-0.810 (Fukushima, 1985). In 18% (w/v) salt, growth of *S. rouxii* occurs at pH 4-5 (Onishi, 1963; Yong and Wood, 1976). Temperature for growth in such high salinity environments is elevated to 40°C (Yong and Wood, 1976; Fukushima, 1985) and growth here involves a process of physiological adaptation (Onishi, 1963; Onishi *et al.*, 1984).

In salt-free medium, the pH range for growth is 3-7 at a temperature between 20-35°C. *S. rouxii* ferments both glucose and maltose in salt-free medium, to alcohol, but ferments only glucose in media containing high salt concentrations (Fukushima, 1985). Onishi (1963, 1984), Yong *et al.* (1980) and Fukushima (1985) have described some of the properties of salt-tolerant *S. rouxii* and its involvement in soy sauce production when salt content is high.

Yong and Wood (1976) observed that viable cells of *S. rouxii* decreased in soy mash, as long as the pH was above 5.5. An increase was observed when the pH dropped below 5.5, after which it remained at a maximum level (Figure 2.13). This was not observed when the mash initially soured with lactic acid at pH 4.5 was inoculated with *S. rouxii*, but there was a lag period of one day. These observations correlated well with a report they reviewed which indicated that "soy-yeasts" could only grow when the pH lay between 4 and 5. Yong *et al.* (1978) showed that optimum yeast growth is achieved in the shortest possible time when the mash is acidified to pH 4.5 with lactic acid. Growth was much less when the medium was acidified with hydrochloric acid.

Brown (1978) observed a special feature of *S. rouxii*. He indicated that, in high salt media, *S. rouxii* produces and accumulates polyalcohols, especially glycerol, as a "compatible solute" which gives the yeasts their resistance to salt, by protecting enzyme activity at low levels of water activity. Onishi and Shiromaru (1984) described the physiological changes occurring during the adaptation of salt-tolerant yeasts. Yong and Wood (1974) reported that furfural in koji is converted to furfuryl alcohol, and together with glycerol, contribute to the flavour of soy sauce.

Torulopsis species have been isolated from older mash (Ho *et al.*, 1984). They are halophilic and are not responsible for the principal alcoholic fermentation, though they convert sugars into alcohols. Sugars other than glucose, for example maltose, under 18% (w/v) salt, are fermented by this species of yeast (Fukushima, 1985). Like *S. rouxii*, they can grow with or without salt, having water activities between 0.975 and 0.840. Rather similar in properties to *S. rouxii*, they are able to grow in as much as 26% (w/v) salt. In 18% (w/v) salt, the growth temperature has an upper limit of 35°C, but in salt-free medium, the temperature range is reduced to 20-30°C. Here again optimum growth depends on pH, such that pH 4-5 is necessary for growth in 18% (w/v) salt medium (Fukushima, 1985).

Torulopsis versatilis and *T. etchellsii*, present in the late stage of fermentation, produce certain components in soy sauce which are not produced by *S. rouxii* (Ho *et al.*, 1984). Yong and Wood (1974) reported the observation by Yokotsuka *et al.* (1967a) that these components produced by *Torulopsis* species gave the soy sauce the characteristic flavour of aged soy mash. The alkyl phenols (Yokotsuka, 1981, 1985) and the aromatic alcohols (Fukushima, 1985), namely 4-ethylguaiacol, 4-ethylphenol and 2-phenylethanol, are the important components formed only by *Torulopsis* species, and are responsible for soy sauce aroma. These species tolerate salt in a manner similar to *S. rouxii* by a physiological adaptation and by producing polyalcohols such as glycerol and erythritol (Fukishima, 1985).

2.4.3 Chemical changes in moromi

Large quantities of glucose are liberated into the brine from the starch contained in the materials through the action of the koji enzymes (Fukushima, 1985). In addition to glucose, other hexoses and pentoses result from the hydrolysis of the carbohydrates. Together, these are metabolised partly into about 1% of lactic acid and other organic acids, including acetic acid and formic acid, by lactic acid bacteria (Yokotsuka, 1985; Fukushima, 1985). The yeasts convert the hexoses and pentoses into 2-3% of ethanol and other flavourous compounds (Yokotsuka, 1985), and what is left of the sugars is present as 2-4% of glucose. Yokotsuka's (1985) review indicates that the enzymatic degradation of the proteins in the raw materials into lower peptides, free amino acids and ammonia stops in 2 or 3 months from the beginning of the mash fermentation, depending on temperature. As well, temperature control contributes to the shortening of the fermentation period.

Aging of the mash, consisting mainly of browning reactions, contributes immensely to the outcome of the final product. Yokotsuka (1985) observed that the colour degree of shoyu mash becomes about double in this latter stage of fermentation, and the pH value stabilizes at 4.8-5.0.

Wood (1982) indicated that, at the end of the fermentation, the moromi possesses all the essential attributes of soy sauce: flavour, aroma, saltiness and colour, as well as an abundance of reducing sugars and amino-nitrogen compounds in the liquid. He also reported that contributions made by the product's salinity, pH value and alcohol content give the sauce its property of resistance to further microbial action.

2.5 Product

Yokotsuka (1960) indicated that a good quality fermented soy sauce contains 1.5 g of total nitrogen and 18 g of NaCl per 100 mL, and appropriate and proper amounts of amino acids, sugar, alcohol, glycerine and organic acids. The sauce, he reported, should have a high buffer capacity, stability, and good aroma, flavour and colour.

2.5.1 Typical composition

2.5.1.1 Nitrogenous compounds

Yokotsuka (1981, 1985) stated that, in order to achieve a palatable taste for a shoyu, about half of its nitrogenous compounds must be free amino acids, and more than 10% of the nitrogenous compounds must be free glutamic acid. He observed in 1960 that the nitrogen compounds consist of about 40-50% amino acids, 10-15% ammonia, 40-50% peptides and peptones, and less than 1% proteins, thereby relating the quality of the sauce to its total nitrogen content.

The Kjeldahl method has been widely used for total nitrogen measurements, however, variations have been observed with the use of catalysts. Yokotsuka (1960) concluded that mercurous compounds are the best catalysts.

The criterion for judging quality of sauce is the ratio of amino nitrogen to total nitrogen (Yokotsuka, 1960). A high ratio indicates high quality. Sasaki and Yokotsuka (1957) observed that the difference between the formol and ammonia nitrogen could be regarded as amino nitrogen. Fukushima (1985) described the formol nitrogen as the α-amino nitrogen content of the sauce, and total nitrogen as the non-proteinous nitrogen. Several reports on soy sauce have identified amino acids, existing mostly in free forms, as well as glutamic and aspartic acids, which exist as conjugated forms. Yokotsuka (1960) cited most of these reports. Of particular interest is glutamic acid, which he showed to be an important flavouring agent and, together with its salt, was observed to be the chief ingredient responsible for the delicious taste of soy sauce. Table 2.7, adapted from Fukushima (1985), lists some of the amino acids present in soy sauce.

Nunomura *et al.* (1978) isolated some nitrogenous compounds from soy sauce. These are the pyrazines which are formed in the Maillard reaction, occurring during the heat treatment of raw materials, aging of moromi, and pasteurization of the raw shoyu. The pyrazines have been identified as flavour components of various heated foods. Yokotsuka (1960) cited the presence of derivatives of nucleic acids, including adenine, cytosine and others, in soy sauce. These were produced by the mould enzymes during the koji stage.

Table 2.7 Amino acid composition of Koikuchi-shoyu.

Amino Acid	Koikuchi-Shoyu (%)
Arginine	2.6
Histidine	2.5
Lysine	6.5
Tyrosine	1.0
Tryptophan	---
Phenylalanine	4.2
Cystine	0.9
Methionine	1.4
Serine	5.3
Threonine	4.2
Leucine	7.3
Isoleucine	4.8
Valine	5.5
Glutamic Acid	22.5
Aspartic Acid	10.5
Glycine	3.9
Alanine	4.4
Proline	6.5
Ornithine	5.7

Adapted from: Fukushima, 1985.

Yong and Wood (1974) cited some reports that maximum soluble protein, polypeptides, peptides and amino acids were obtained from koji having equal parts of raw materials. He also indicated that mixing koji with more water caused a better utilization of the total nitrogen of the raw material, although this may result in some undesirable effect on the composition of the sauce. The report also noted that a lower salt content ensured a better utilization of total and amino nitrogen as well as a better fermentation. Work by other investigators (Yong and Wood, 1974), on the composition of mashes aged differently, showed that free glutamic acid could be used to determine the optimum aging period, since glutamic nitrogen was at its maximum after 10 or 11 months of fermentation.

2.5.1.2 Sugars and alcohols

Yokotsuka (1960) listed the sugars present in soy sauce as arabinose, xylose, glucose and galactose, and stated that the 3-5% reducing sugar required in good quality sauce is expressed as glucose equivalent. In his review article in 1985, Yokotsuka indicated that the carbohydrates are hydrolysed to hexoses and pentoses, which are partly metabolised by the microorganisms and that the final mash contains about 2-4% glucose and trace amounts of xylose.

Fukushima (1985) reported that soy sauce contains monosaccharides made up of 0.62 mannose, 0.77 arabinose, 1.72 galactose, 0.55 xylose, and 20.50 glucose (expressed in mg/mL). In addition, he listed disaccharides, oligosaccharides and polysaccharides, and expressed the total amount of sugar as 4.45% glucose.

Also found in the sauce were two sugar alcohols, glycerol and mannitol, and a non-reducing oligosaccharide (Yokotsuka, 1960). Ethanol and some other alcohols are present (Fukushima, 1985). Nunomura *et al.* (1980) identified 15 alcohols in shoyu flavour components.

Glycerol or glycerin differentiates a soy sauce prepared with whole soybeans from that made with defatted soybeans. Yokotsuka (1960) reported that 0.4-0.5% glycerol is produced in sauce made from defatted meal, while 1.0-1.2% results from whole beans. Onishi (1963) and Onishi and Shiromaru (1984) have also reported that, as a result of physiological adaptation to its high saline environment, *S. rouxii* produces some glycerol. *S. rouxii* metabolises glucose in moromi to ethanol, thereby giving the sauce its alcoholic content of 2-2.5%. Yokotsuka (1960) concluded that 0.5% or more of glycerol by weight in soy sauce is organoleptically detectable because of its sweet taste. Yong *et al.* (1981) also suggested that the presence of ethanol and glucose in the moromi mash enhanced ester formation by the yeast. Ethyl acetate and other esters synthesized are responsible for the characteristic bouquet and flavour of the mature soy sauce.

The sugar content in soy sauce has been determined by different methods, and Yokotsuka (1960) listed some problems associated with these methods. Yong and Wood (1977a) employed Sumner's 1925 method. In 1985, Osaki *et al*, determined the residual glucose in their soy sauce, produced with immobilized whole cells, by a glucose analyzer, and the alcohol was monitored by a Teflon tubing method with flame ionization detector or gas sensor.

2.5.1.3 Acids and related compounds

In raw soy sauce, lactic acid accounts for 85% of the organic acids and acetic acid is 8% (Yokotsuka, 1960). Investigators have observed that the organic acids are important to the aroma, flavour, colour and storage quality of soy sauce. Yokotsuka (1960) reported that the major acids found in the sauce include acetic, lactic, succinic and phosphoric acids. Their salts, however, taste bitter. Table 2.8, taken from Yokotsuka (1986), shows the organic acids as found in shoyu and analysed by Ueda *et al.* (1958).

Yokotsuka (1960) also reported that the amounts of citric and malic acids present at the start of the brine fermentation decreased throughout the fermentation period. Propionic and formic acids are also present in soy sauce. Usually, formic acid is the main organic acid in chemical soy sauce and, together with levulinic acid, differentiate chemical soy sauce from fermented soy sauce (Fukushima, 1985).

Acetic acid is produced by *P. halophilus* (Noda *et al.*, 1981) in addition to lactic acid. It is also produced by *S. rouxii*, when grown at the initial stage where pH of moromi is about 6.5-7 (Yong *et al.*, 1978). The two organisms metabolise glucose present in the mash to the

Table 2.8 Content of major organic acids in shoyu.

Organic Acid	Pasteurized Shoyu (mg/100 mL)
n-Butryic	0.5
Propionic	4.0
Levulinic	4.4
Acetic	126.2
Pyruvic	11.9
Formic	6.2
α-ketobutyric	0.2
Lactic	1156.6
Succinic	49.8
Pyroglutamic	110.6
Glycolic	9.9
Malic	Trace
Citric	Trace

Adapted from: Yokotsuka, 1986.

acids, but *P. halophilus* also metabolises citric acid present in soybeans to lactic, acetic and formic acids (Fukushima, 1985), and also metabolises malic acid (Kanbe and Uchida, 1982). Nunomura *et al.* (1980) identified 13 organic acids as flavour components of soy sauce.

Yokotsuka (1960) reported that titratable acidity was effective in ameliorating the salty and acidic tastes and in promoting the stability of soy sauce. Onaga *et al.* (1957) indicated that total acidity appeared to be important to flavour acceptance. Samples of sauce which had high total acidity were ranked higher in flavour.

2.5.1.4 Colour

According to Hesseltine and Wang (1972), a perfect fermented moromi should have a bright reddish-brown colour. This is important since it is associated with flavour (Yokotsuka, 1960).

Yokotsuka's (1960) report also mentions that the colour development of soy sauce is chiefly due to the browning reaction. Many types of flavouring are made by this reaction, and the browning reaction takes place among many kinds of important flavouring ingredients, such as amino acids and sugars.

Yong and Wood (1977a) cited a report by Oba *et al.* (1974), who observed the presence of tyrosinase in rice koji and correlated the browning occurring in the koji with tyrosinase level, implying an enzymatic browning. On the other hand, Yong and Wood (1977a) were not able to detect any tyrosinase in soy koji, and this was in agreement with an observation by some other investigators. Thus, although the colour of moromi mash deepens during fermentation and maturation, this could probably be due to non-enzymatic reactions (Wood, 1982).

Non-enzymatic browning of soy sauce occurs also during pasteurization process and storage. In both cases the browning was developed on heating or spontaneous oxidation. Oxidative browning (during storage or after opening) is undesirable since it is accompanied by the deterioration of taste and flavour (Hashiba, 1972). Hashiba (1973) observed that amino acids and sugars were highly related to the browning of soy sauce on oxidation as well as heating, but Hashiba (1972) also pointed out that, while both sugars and amino acids were involved in the oxidative browning, heat browning mainly involved sugars with the amino acids, probably acting as catalysts. Hashiba (1975, 1976, 1978) observed that Amadori compounds played an important role in oxidative browning of the sauce, and isolated 5 such compounds. He observed that their formation was by reaction of glucose and neutral amino acids. These five Amadori compounds are fructose-glycine, fructose-alanine, fructose-valine, fructose-isoleucine and fructose-leucine, and they exhibit remarkable browning in the presence of oxygen and iron, but darkened very little without oxygen or iron (Hashiba, 1978). Hashiba *et al.* (1981) included fructose-proline as the sixth Amadori compound isolated from soy sauce. The presence of these compounds and, therefore, their isolation from the sauce, is explained to be that they are present in large amounts or that they are more stable. Though not isolated, it is considered that Amadori compounds from pentose are present in soy sauce. The contribution of pentose to oxidative browning was estimated to be 75%, whereas hexose contributed 25%. Hashiba *et al.* (1981) also observed that the Amadori compounds derived from peptides and hexose were more reactive in non-oxidative (heat) and oxidative browning than those derived from amino acids and hexose, but less reactive than the Amadori compounds from pentose.

The red colour of soy sauce originates from the sugar moiety of Amadori compounds (Hashiba *et al.*, 1981). They noted that any increase in side chain of amino acids composing the Amadori compounds decreased the colour intensity.

In his review paper in 1986, Yokotsuka indicated that 50% of the colour of Koikuchi shoyu is formed during fermentation and aging of mash, and the remaining 50% during pasteurization. The browning occurring for the former is mostly due to Maillard reactions which, as previously mentioned, occur heat-dependently between amino compounds, such as amino acids, peptides and proteins, and carbonyl compounds, represented by sugars. Yokotsuka (1986) also cited a report which indicated that soybeans and wheat both contributed to the heat-dependent browning of soy sauce by 60% and 40%, respectively. Also contributing to browning reaction, as intermediates, either in absence or presence of air, are 3-deoxy-D-glucosone, 3-deoxy-xylosone, furfural and acetaldehyde which, being carbonyl compounds, react with the amino acids present in soy sauce.

The type of lactobacillus used in the salty mash fermentation also contributes to the colour of soy sauce. A detailed account on the colour of soy sauce is given in Yokotsuka's (1986) review.

2.5.1.5 Flavour components and quality evaluation

Yokotsuka (1986) reported that nearly 300 volatile flavour compounds in the fragrance of Koikuchi shoyu have been identified by Japanese investigators. The compounds or components include 51 carbonyls, 24 organic acids, 41 esters, 31 alcohols, 3 acetals, 11 sulphur and 36 nitrogenous compounds, 17 phenols and 62 others. He observed that the most important fragrance characteristic existed in the weak acidic fraction of shoyu.

Nunomura *et al.* (1976, 1978, 1980, 1984) studied the volatile flavour components of soy sauce. They fractionated the soy sauce into basic, acidic and neutral components, then isolated and identified the important flavourous compounds present.

Yokotsuka (1981) observed that, on neutralizing soy sauce with alkali, the characteristic fragrance of the sauce disappeared and did not return after it was re-acidified. This, and the fact that the strongest fragrance was observed in the phenolic fraction, led investigators to isolate the important flavourous compounds from the weak acidic fraction of soy sauce. The presence of 0.5-2.0 ppm of 4-ethylguaiacol was observed to have the taste characteristic of fermented shoyu and it also ameliorated the salty taste of the sauce (Yokotsuka, 1985). Together with p-ethylphenol, 4-ethylguaiacol and 2-phenylethanol are produced by ***Torulopsis versatilis*** and ***T. etchellsii*** (Fukushima, 1985). An investigation in 1960, into shoyu mashes, showed that the organoleptically good mashes contained the ***Torulopsis*** species (Yokotsuka, 1985).

Hydroxy furanones, found in the acidic fraction, were observed to have caramel-like flavour. Included here are 4-hydroxy-2(5)-ethyl-5(2)-methyl-3(2H)-furanone (HEMF), 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF) and 4-hydroxy-5-methyl-3(2H)-furanone (HMF). With a low threshold value of 0.04 ppb and its presence in soy sauce in amounts of 50-100 ppm, HEMF makes the greatest contribution to the good aroma of the acidic fraction

(Nunomura *et al.*, 1980). Aside from having an intense and fruity aroma peculiar to soy sauce flavour, the content of HEMF correlated positively to the quality of shoyu (Nunomura *et al.*, 1980), and it was concluded that HEMF is the most important constituent of the characteristic flavour of shoyu.

Pyrazines were found to be the major constituents in the basic fraction, which gave the so-called "heated" flavour to soy sauce (Nunomura *et al.*, 1978). Formed mostly by the Maillard reaction, they are considered to occur during the heat treatment of raw materials, aging of mash, and, more significantly, during the pasteurization of raw sauce. According to Nunomura *et al.* (1978), the flavour concentrate from which the basic compounds are removed has a heavy note to some extent, organoleptically, and the basic compounds play the role of reducing that heavy note of shoyu aroma, rather than participating in the essential qualities of shoyu flavour. The basic compounds can thus be judged to be indispensable to the shoyu flavour.

Phenylacetaldehyde, present in the neutral fraction, was observed to contribute greatly to the aroma of soy sauce (Nunomura *et al.*, 1984). The fraction also contained 2-phenylethanol, which is produced by *Torulopsis* species (Fukushima, 1985).

Reporting on work done by some investigators on the flavour evaluation of Koikuchi shoyu, Yokotsuka (1985) pointed out that the fragrance of a fermented shoyu is roughly proportional to its content of ethanol, which is produced by yeast fermentation. It was observed that 11 chemical components, including alcohol, degrees Baumé (density), sodium chloride, reducing sugar, total nitrogen, formol nitrogen, titratable acidity and pH contributed only 46.3% to the preference for shoyu, with the alcohol content having the highest value. Eleven aspects of odourous characteristics contributed 96.5% to the preference for odour of shoyu, while 9 characteristics regarding taste contributed 97.6% to preference for the taste of shoyu. Good after-taste, pure taste and palatable taste had the highest positive correlation coefficients, while too sweet, too sour, abnormal taste and lack of harmony and body were the opposite. In conclusion, the investigators stressed that a good flavour shoyu must be made by a totally fermentative method, free from disagreeable odour derived from bacterial contamination, must be well fermented by yeasts, and must have well-balanced chemical components. Thus, an organoleptically preferable shoyu has not only good harmony of taste components, such as salty, acidic, sweet, bitter and delicious, but also good aroma.

2.6 Substitute Raw Materials for Sauce Production

According to Yong and Wood (1974), several attempts had been made at producing soy sauce from raw materials other than soybeans and wheat. Though these attempts proved unsuccessful, it is of great interest to researchers in product development to make use of raw materials, which otherwise have had no significant use for human consumption, into sauce products which could be substituted for soy sauce, or could remain as additional sources of amino acid-peptide hydrolysates.

Yong and Wood (1974) cited Church's experiment in 1923, in which peanut press cake was used instead of soybean and wheat mixture to make the sauce. The sauce obtained was evidently peanut sauce, since the taste of peanut was greatly retained. The authors reported in this paper that, since control of bacterial growth and activity in such a process would be extremely difficult, fermentation of peanut press cake on an industrial scale would not be easy. They also reviewed the work by a Japanese invesigator. Apparently, during the Second World War, the Japanese were desperate enough to try to produce soy sauce from garbage. Not long afterwards, in 1949, Oda *et al.* prepared a "substitute soy sauce" using acorns and wheat with an *Aspergillus* culture which produced tannase. In 1964 the production of soy sauce from soybean hull was attempted by Kato and Matsumoto (Yong and Wood, 1974).

By 1966, as reported by Yong and Wood (1974), copra meal had been found as a cheap source of proteinaceous material by Baens-Arcega, who then claimed to have successfully utilized it, together with soybeans, in a ratio of 1:1, for the mould process of manufacturing soy sauce.

Luksas (1971a,b) suggested the production of sauce from whey by fermenting with *Saccharomyces lactis* and *S. cerevisiae*. More recently, though, having investigated the possibility of bringing a high protein crop into acceptable form of human food, Ooraikul *et* *al.* (1980) utilized rapeseed meal, a by-product of oil extraction process and a cheap source of protein, to produce a sauce comparable in its acceptability to a commercial soy sauce.

As a result of the success of the preliminary study on the utilization of rapeseed meal to produce a sauce similar to soy sauce, attempts have been made at improving the quality of the sauce (Ma and Ooraikul, 1986).

2.6.1 Rapeseed meal and sauce production

Originally called rapeseed meal, this by-product of oil extraction process of rapeseed, containing little or no erucic acid and very low glucosinolates, is now referred to as canola meal.

Containing as much as 40% protein and rated as the best vegetable protein by Sosulski and Sarwar (1973), Ooraikul *et al.* investigated the feasibility of its use as a raw material to replace soybean meal for sauce production. Table 2.9 compares the proximate and amino acid compositions of canola meal and soybean meal.

Ooraikul *et al.* (1980) produced the sauce by using both the Conventional (adapted from Umeda *et al.*, 1969) and Semi-chemical (adapted from Hesseltine and Wang, 1972) methods. Results from the two methods proved that canola meal could be substituted for soybean meal in sauce production. Though the semi-chemical approach shortened the fermentation period to about one month, the use of hydrochloric acid to prehydrolyse the canola meal was observed to be economically unsound on an industrial scale. For instance, the high concentration of acid is corrosive, and to be used industrially would require processing equipment which is resistant to the acid, in which case production costs would be too high to be competitive. The process would require the use of food grade acid and alkali, which are expensive, and the use of the acid would result in the undesirable compounds peculiar to chemically hydrolysed meal. Ooraikul *et al.* (1980) observed that yeast fermentation did not occur for this semi-chemical process, and this deprived the sauce of the aromas and flavour characteristic of soy sauce.

Table 2.9 Proximate and amino acid compositions of canola meal and soybean meal.

	Canola Meal	Soybean Meal
Proximate Composition		
Moisture	7.49	11.00
Crude Fibre	11.09	7.3
Ether Extract	3.78	0.8
Protein (N x 6.25)	37.96	45.01
Amino Acid Composition (in protein %)		
Alanine	4.56	4.20
Arginine	6.11	6.44
Aspartic Acid	8.03	11.20
Cystine	1.23	0.65
Glutamic Acid	16.69	18.00
Glycine	4.96	4.60
Histidine	2.81	2.40
Isoleucine	3.98	4.69
Leucine	6.97	7.49
Lysine	5.98	6.22
Methionine	1.78	1.40
Phenylalanine	4.01	4.80
Proline	7.00	4.89
Serine	4.39	5.00
Threonine	4.50	3.80
Tryptophan	1.16	1.20
Tyrosine	2.46	2.80
Valine	5.11	5.00

Adapted from: "Canola Meal for Livestock and Poultry", June 1981; D.R. Clandinin, Canola Council of Canada, Publication No. 59.

As a result of these problems, an improvement on the semi-chemical process was researched.

2.6.2 Improved short-fermentation method

In a bid to solve the problems encountered in the procedure used by Ooraikul *et al.* (1980), the acid hydrolysis of canola meal was eliminated and replaced by a moderate hydrolysing agent in the form of a proteolytic enzyme, alcalase 0.6L (Ma, 1985). An endo-protease of the serine type, alcalase 0.6L was used to prehydrolyse the canola meal, after which the digested meal was mixed with roasted wheat and inoculated with *Aspergillus oryzae* and *A. sojae*. The mixture was then allowed to undergo the natural fermentation. The process is outlined in Figure 2.14.

2.6.3 Result of enzyme-hydrolysed canola meal in sauce production

The conditions under which alcalase 0.6L was used to prehydrolyse canola meal resulted in a total soluble nitrogen (TSN) value which accounted for over 60% of nitrogen yield of the canola sauce. Although the canola sauce prepared with a combination of *A. oryzae* and *A. sojae* produced the greatest TSN value of 1.34% and nitrogen yield of about 73.38%, it had the lowest amino nitrogen (AN) content, indicating that other nitrogenous compounds were present but did not contribute significantly to the taste or aroma of the sauce. As evidence of a good quality sauce, as reported by Hesseltine and Wang (1972), the AN/TSN ratio must be about 50%. Ma and Ooraikul (1986) indicated that most of the canola sauces had AN/TSN ratios greater than what was observed in the Kikkoman sauce used as control, except the sample which was made from the combination of Aspergilli (Table 2.10).

The amounts of the amino acids present in canola sauces (Table 2.11) were mostly lower than those found in the Kikkoman sauce (Ma and Ooraikul, 1986). Cystine and methionine concentrations were higher in canola sauces than in the Kikkoman sauce because canola meal *per se* is higher in sulphur-containing amino acids, such as the above, and lower in lysine, while the other amino acids are comparable in content to that in soybean meal. Some amino acids were also present in the enzyme hydrolysate. This indicates that alcalase

Table 2.10 Total soluble nitrogen (TSN), nitrogen yield, amino nitrogen (AN) and AN/TSN ratio of canola sauces, Kikkoman shoyu and enzyme hydrolysate of canola meal (averages of two replicates).

Samples[1]	TSN (w/w) (%)	Nitrogen[2] Yield (%)	AN (w/v) (%)	AN/TSN
CS1	1.31	71.74	0.80	61.06
CS2	1.16	63.53	0.70	60.34
CS3	1.34	73.38	0.66	48.50
CS4	1.12	61.34	0.73	65.18
KS	1.38	73.70[3]	0.70	58.72
EH	0.85	46.54	0.64	75.29

[1] CS1 = canola sauce prepared with *A. oryzae* and initial moromi pH of 5.5.
CS2 = canola sauce prepared with *A. sojae* and initial moromi pH of 5.5.
CS3 = canola sauce prepared with *A. oryzae* and *A. sojae* and initial pH of 5.5
CS4 = canola sauce prepared with *A. oryzae* and initial moromi pH of 6.5.
KS = soy sauce produced by Kikkoman Shoyu Co., Ltd., Japan.
EH = enzyme hydrolysate of canola meal (with Alcalase 0.6L) prior to *Aspergillus* fermentation.

[2] Nitrogen yield = TSN in sauce/total nitrogen content in raw materials (4.565 g/100 g).

[3] From Hesseltine and Wang (1978).

Adapted from: Ma and Ooraikul, 1986.

Table 2.11 Amino acids in canola sauce, Kikkoman shoyu and enzyme hydrolysate of canola meal (mMole/mL, average of triplicate determinations).

Amino acid	CS1	CS2	CS3	CS4	KS	EH
Aspartic acid	24.40	34.50	35.50	39.50	65.70	4.35
Threonine	14.40	16.70	24.25	19.85	30.60	1.95
Serine	16.45	29.45	31.40	31.50	52.35	4.30
Glutamic acid	61.85	83.40	87.75	76.80	86.55	9.65
Proline	20.80	25.25	34.25	21.85	52.00	6.35
Glycine	29.35	25.10	28.90	28.90	37.05	3.40
Alanine	37.05	40.00	47.75	43.95	55.65	6.75
Cystine	6.20	7.35	8.75	7.00	3.40	4.90
Valine	29.80	38.35	43.50	38.80	55.95	6.70
Methionine	13.60	12.10	17.15	11.90	10.05	5.85
Isoleucine	19.05	23.55	26.55	23.90	41.30	4.70
Leucine	34.45	41.75	29.10	40.15	65.10	8.15
Tyrosine	11.20	13.75	16.15	13.15	6.90	1.70
Phenylalanine	16.45	18.65	21.55	17.00	31.05	3.95
Lysine	19.35	23.85	29.10	24.05	49.05	1.45
Arginine	17.65	25.70	30.55	25.75	21.75	2.00

For an explanation of abbreviations used, see Table 2.10.
Adapted from: Ma and Ooraikul, 1986.

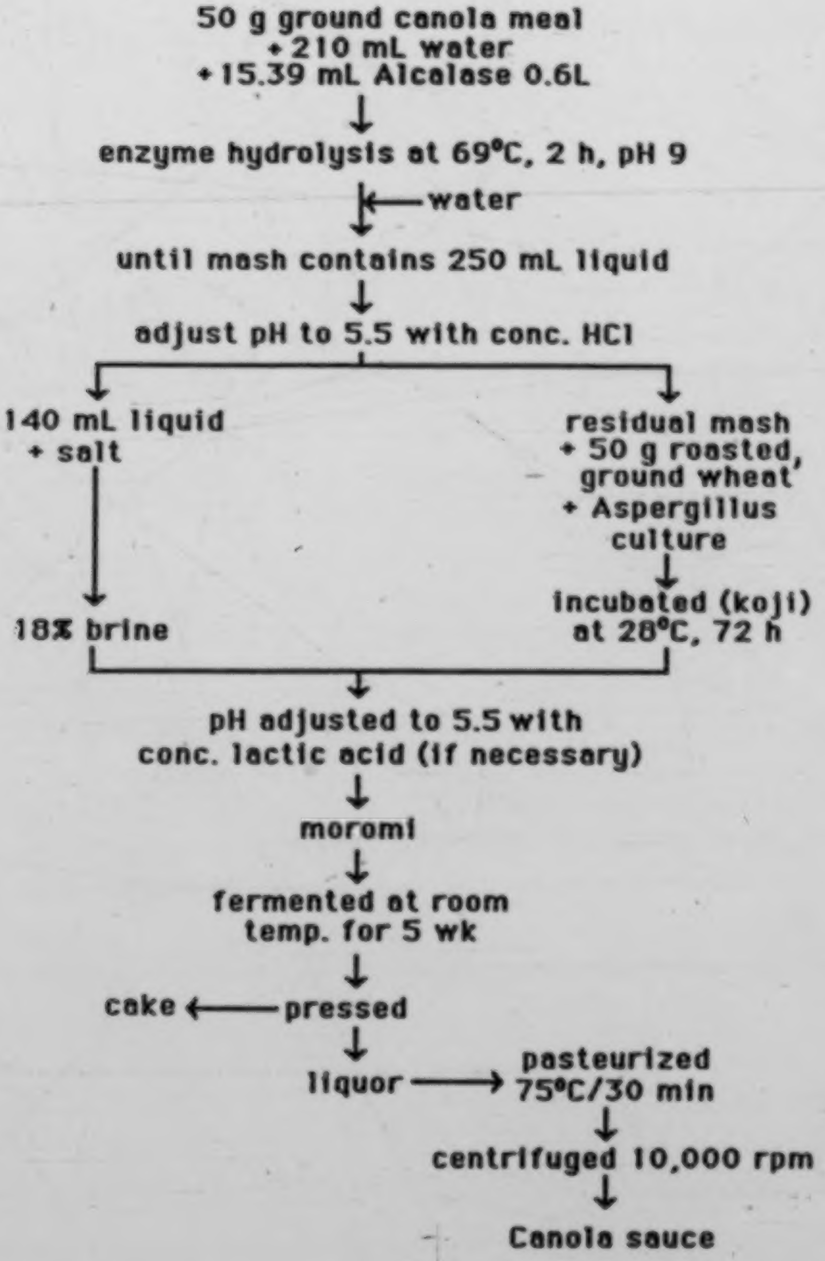
50 g ground canola meal
+ 210 mL water
+ 15.39 mL Alcalase 0.6L
enzyme hydrolysis at 69°C, 2 h, pH 9
water
until mash contains 250 mL liquid
adjust pH to 5.5 with conc. HCl
140 mL liquid
+ salt
18% brine
residual mash
+ 50 g roasted, ground wheat
+ Aspergillus culture
incubated (koji) at 28°C, 72 h
pH adjusted to 5.5 with conc. lactic acid (if necessary)
moromi
fermented at room temp. for 5 wk
cake
pressed
liquor
pasteurized 75°C/30 min
centrifuged 10,000 rpm
Canola sauce

Figure 2.14 Flow chart of enzymatic production of canola sauce (adapted from: Ma, 1985).

hydrolysis not only breaks down crude proteins to larger peptides, but also continues the conversion of some peptides to amino acids. The presence and amount of glutamic acid in the sauce is highly significant since it is important as a flavouring agent in soy sauce. Yokotsuka (1960) has reported Udo's (1931b) conclusion that the chief ingredient responsible for the delicious taste of soy sauce was glutamic acid and its salt.

Like Yong and Wood (1976), Ooraikul *et al.* (1980) also reported a two-step increase of total soluble solids (TSS) in the conventional method of sauce production. This was attributed to the two-stage fermentation, involving lactic bacteria growth initially, then the growth of yeasts. Yeast growth coincided with a mash of pH below 5. On the other hand, Ooraikul *et al.* (1980) observed that there was only one rise of TSS in their acid hydrolysed procedure, which remained unchanged to the end of the fermentation period. This observation was also reported by Ma (1985). Their conclusions were similiar, in that there was no yeast fermentation to give a second rise and, most importantly, yeast fermentation could not be initiated at pH above 5. The changes in TSS and pH were due to the conversion of the proteins and sugars present in the mash to amino acids, organic acids and other compounds (Figures 2.15 and 2.16).

As a result of the improper development of lactic acid and yeast fermentations, the titratable acidity found in the canola sauces was slightly lower than that observed in the Kikkoman sauce. Yet, based on the qualitative and quantitative characteristics of organic acids in the sauces, Ma and Ooraikul (1986) indicated that the quality of the canola sauce appeared to fall between that of naturally fermented and chemical soy sauces. As indicated in Table 2.12, the organic acids in canola sauces were generally less than what was found in Kikkoman sauce.

The salt and reducing sugar contents of canola sauces compared favourably with Kikkoman sauce (Table 2.13), but greater amounts of non-reducing sugar were present in the former, probably due to the shorter fermentation period and, possibly, the use of different raw materials.

Table 2.12 Total acidity (meq. NaOH/100 mL) and organic acid concentration (mg/100 mL) of canola sauces, Kikkoman shoyu and enzyme hydrolysate of canola meal (averages of two replicates).

	CS1	CS2	CS3	CS4	KS	EH
Total acidity	24.40	16.65	23.45	29.95	33.35	16.30
Citric acid	8.60	6.50	12.46	4.60	7.13	1.48
Isocitric acid	2.65	0.66	2.18	1.71	3.03	-
Pyruvic acid	0.14	0.07	0.15	0.13	0.18	-
Unknown 1	++	+	++	+	-	-
Malic acid	2.98	1.44	3.07	2.51	4.75	-
tr-aconitic acid	0.03	+	0.02	0.02	-	-
Unknown 2	++	+	++	++	++	-
Glyoxylic acid	0.08	-	-	-	-	-
Succinic acid	0.74	-	0.74	0.52	+	-
Formic acid	14.12	16.76	U/E	6.86	16.87	0.57
Lactic acid	36.01	63.78	U/E	74.58	87.86	-
Propionic acid	10.90	2.20	3.80	7.60	48.49	-

For an explanation of abbreviations used, see Table 2.10.
++ = significant amount; + = present; - = absent; U/E = unable to estimate due to overlap.
Adapted from: Ma and Ooraikul, 1986.

Table 2.13 Sugar and salt contents of canola sauces, Kikkoman shoyu and enzyme hydrolysate of canola meal (averages of two replicates).

Sample	Reducing sugar (% glucose)	Non-reducing sugar (% sucrose)	Total sugar (%)	Salt (% NaCl, w/v)
CS1	8.81	6.58	15.39	19.22
CS2	7.43	1.26	8.69	18.72
CS3	8.77	2.08	10.85	19.85
CS4	9.67	0.49	10.16	20.37
KS	6.76	0.95	7.71	18.12
EH	trace	trace	trace	trace

For an explanation of abbreviations used, see Table 2.10.
Adapted from: Ma and Ooraikul, 1986.

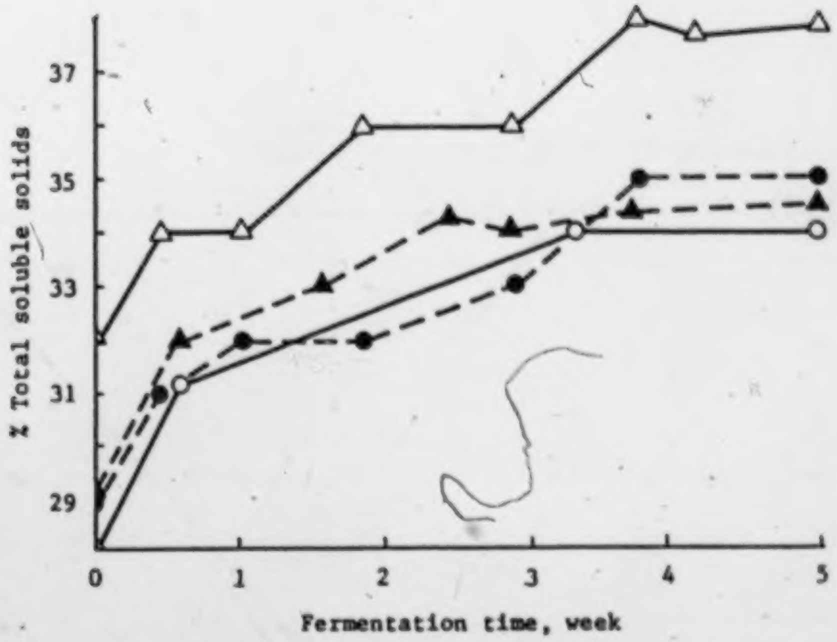

Figure 2.15. Change in total soluble solids of the mash during the moromi stage of canola sauce fermentation (adapted from: Ma and Ooraikul, 1986).
(△) CS1, *A. oryzae* made koji, initial moromi pH 5.5
(▲) CS2, *A. sojae* made koji, initial moromi pH 5.5
(○) CS3, *A. oryzae* and *A. sojae* made koji, initial moromi pH 5.5
(●) CS4, *A. oryzae* made koji, initial moromi pH 6.5

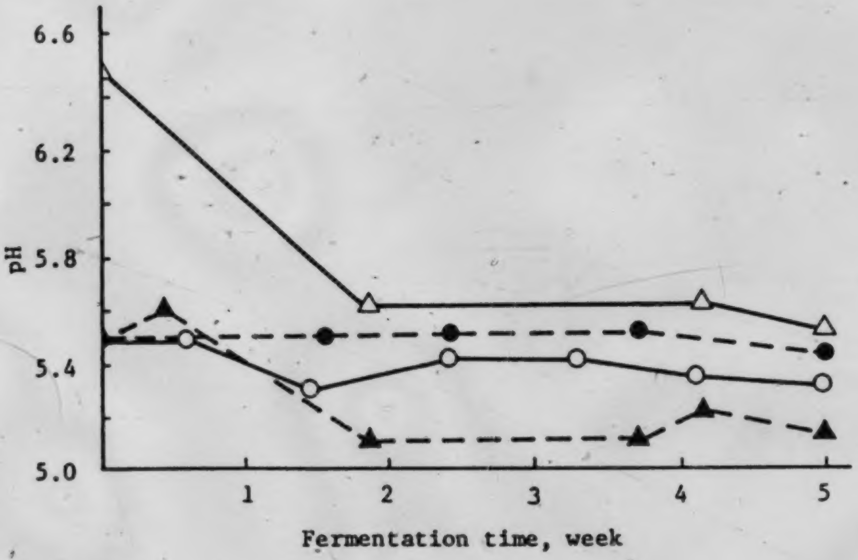

Figure 2.16. Change in pH of the mash during the moromi stage of canola sauce fermentation (adapted from: Ma and Ooraikul, 1986).
(▲) CS1, *A. oryzae* made koji, initial moromi pH 5.5
(●) CS2, *A. sojae* made koji, initial moromi pH 5.5
(○) CS3, *A. oryzae* and *A. sojae* made koji, initial moromi pH 5.5
(△) CS4, *A. oryzae* made koji, initial moromi pH 6.5

The colour difference between canola sauce and Kikkoman sauce was attributed to the different conditions, especially temperature and time, employed in the production and pasteurization of the sauces and also to the constituents of the different raw materials. It was observed that the colour of canola sauce was slightly darker than Kikkoman sauce, and it had a yellowish tinge, while the Kikkoman was more reddish.

Even though the sensory evaluation results were inconclusive and difficult to interpret, the overall acceptance suggested that the canola sauce was inferior to Kikkoman sauce but comparable to Chinese soy sauce. The report also indicated that the canola sauce lacked the myriad aromas typical of high quality soy sauce, a difference which was attributed to the deficiency in acid and sugar fermentations during the moromi stage of sauce production. Although the taste of canola sauce was judged to be quite similar to soy sauce, an improvement in organoleptic quality was necessary.

3. EXPERIMENTAL

3.1 Culturing of Microorganisms

3.1.1 Revitilization of stock mould cultures

3.1.1.1 Materials

I. Microorganisms

Aspergillus oryzae 14895 (NRRL 1989) and *Aspergillus soja* 16320 (American Type Culture Collection [ATCC], Rockville, MD). Both cultures were maintained on Potato Dextrose Agar.

II. Media

Potato Dextrose Agar [PDA] (Difco Laboratories, Detroit, MN); Peptone Water.

3.1.1.2 Equipment

(a) AMSCO Lab/Isothermal Autoclave (American Sterilizer Co., Erie, PA).

(b) Low temperature incubator (Thelco. Precision Scientific, GCA Corp., Chicago, IL).

(c) Roux bottles and common culturing facilities.

3.1.1.3 Culture Method

Mould mycelia were aseptically transferred from roux bottles into separate sterile test tubes containing about 5 mL of sterile peptone water, and mixed thoroughly. Approx. 0.1 mL portions of the suspensions of both mould cultures were transferred aseptically onto separate PDA plates and incubated at 37°C for 5 d until profuse sporulation occurred. Spores were scraped and the aseptic transfer process to peptone water was repeated. A loopful of spores was streaked on PDA plates and incubated at 37°C for 48 h. A single colony from each type of mould plated was then subcultured 3 times, and transferred onto slants. These were incubated and maintained thereon.

Five mL peptone water was added to slants of both moulds and the mycelia were loosened with a sterile spatula into the liquid phase. One mL of the suspensions from both slants was pipetted aseptically onto separate pre-prepared PDA beds in sterile roux bottles, and the suspensions spread smoothly over the agar bed with a sterile glass spreader. The roux bottles were plugged with sterile dispo plugs and incubated at 37°C for 48 hr.

3.1.2 Rehydration of freeze-dried bacteria and yeast cultures

3.1.2.1 Materials

I. Microorganisms

Pediococcus halophilus ATCC 21786, *Saccharomyces rouxii* ATCC 13356, and *Torulopsis versatilis* ATCC 20191.

II. Media and reagents

(a) Sodium Acetate Medium 1 (SAM 1), broth and agar (formulation described in ATCC Catalogue, Rockville, MD). The basic components of SAM1 broth are: (33 g) Sodium Acetate, (10 g) Glucose, (3 g) Yeast Extract, (10 g) Peptone, (5 g) K_2HPO_4, (5 g) NaCl and distilled water to make up to 1 L. (15 g) Agar was added to the broth composition to make SAM 1 Agar.

(b) YM broth and Agar (Difco Laboratories, Detroit, MN).

(c) Sterile 1 M HCl.

3.1.2.2 Equipment

Controlled Environment Incubator Shaker (New Brunswick Scientific Co., Inc., Edison, NJ).

3.1.2.3 Culture methods

Freeze-dried cultures were aseptically removed from the vials in the following manner:

For the double-vial preparations, the tip of the outer vial was heated in a flame, and a few drops of water were squirted on the hot tip to crack the glass. A file was used to strike the tip, after which the inner vial was removed. With sterile forceps, the cotton plug was removed from the inner vial.

Opening the single-vial preparation involved scoring the ampoule once with a sharp file, about an inch from the tip. The ampoule was then disinfected with alcohol-dampened gauze, wrapped with the same gauze and the tip broken at the scored area.

Sterile SAM 1 broth and YM broth, which had been prepared before hand, were used to rehydrate the freeze-dried cultures. SAM 1 broth (0.4 mL) was aseptically added to the freeze-dried *Pediococcus halophilus* culture with a sterile pipette, mixed thoroughly and then transferred into a sterile test tube containing 5 mL of SAM 1 broth, pH 6.9. A drop of the original mixture of culture was punched into an agar slant. Both slant and inoculated broth were then incubated at 30°C for 3 to 4 days. The culture, maintained on the slant, was then stored at 4°C.

Both yeasts, *Saccharomyces rouxii* and *Torulopsis versatilis* were rehydrated in a similar manner, but in sterile YM broth, and maintained on the surface of YM agar slants. The pH of the broth was kept at 4.5. Inoculated broth and slants were also incubated at 26°C for 4 days, in a shaking incubator. The culture, maintained on the slant, was stored at 4°C.

All the growing cultures of *P. halophilus*, *S. rouxii* and *T. versatilis* were subcultured 7 times until the growth was profuse.

Cultures of microorganisms, maintained in their respective media, were subcultured monthly.

3.1.3 Adaptation of microorganisms to high saline environments

3.1.3.1 Training procedure

Broths of both media were made with increasing salt content of 3, 6, 9, 15 and 18% (NaCl, w/v). The pH of SAM 1 broth was adjusted to 6.9-7.0 with sterile 1 M NaOH, while the pH of YM broth was adjusted to 4.5 with sterile 1 M HCl.

One mL each of the cultured microorganisms (section 3.1.2.3) was inoculated separately into 6 test tubes. Each test tube contained 5 mL of broth with the salt concentrations indicated above. All the cultures were incubated at 30°C. The bacteria cultures were incubated statically for one week, while the yeast cultures were incubated for 5 days in a shaking incubator.

Cells growing in the 18% NaCl (w/v) media were thereafter transferred into fresh media, but with same salinity. Subculturing of cells growing in 18% salt medium was done 7x, at a weekly interval. The trained cultures were maintained in 18% salt medium, incubated for 7 d in the case of *P. halophilus* and 5 d in the case of *S. rouxii* and *T. versatilis*, then stored at 4°C. The 'trained' bacteria culture, maintained in 18% salted SAM 1 broth, and the 'trained' yeasts, also maintained in 18% w/v NaCl-YM broths, were subcultured bimonthly.

3.1.4 Growth of 'trained' microorganisms

3.1.4.1 Equipment

(a) Spectronic 20 (Bausch and Lomb).
(b) Sterile 250 mL side-arm erlenmeyer flasks.
(c) Petri-dishes; dispo plugs.

3.1.4.2 Procedure

Five mL each of 'trained' *P. halophilus*, *S. rouxii* and *T. versatilis* cultures were transferred into 3 sterile 250 mL side-arm erlenmeyer flasks, one containing 50 mL of 18% NaCl-SAM 1 broth, and the other two containing 18% NaCl-YM broth. The pH of SAM 1 broth was adjusted to 6.9-7.0 and YM broth to pH 4.5. The flasks were plugged with sterile non-absorbent dispo plugs which had been wrapped with aluminum foil, and incubated at 30°C. *P. halophilus* was incubated statically for 11 d, while the yeasts were incubated in a shaking incubator for 6 days.

The outside of the side-arms on the flasks were wiped clean with alcohol, the contents in the flasks shaken thoroughly to mix the cultures, and the flasks tilted to obtain some culture in the arms. The side-arms were then inserted into the cell slot of the spectrophotometer and the optical densities read at 550 nm. Uninoculated 18% NaCl-SAM 1 broth and 18%

NaCl-YM broth, in similar flasks, were used as reference blanks.

On each day of OD measurement, 1 mL of inocula from all 3 'trained' cultures was aseptically pipetted into 3 separate sterile test tubes having 9 mL of 0.1% sterile peptone water and containing 18% w/v NaCl. Serially diluted bacteria cultures (0.1 mL) were plated on both basic and salt-containing SAM 1 Agars and allowed to dry, after which molten SAM 1 agars with similar compositions were poured over to create microaerophilic conditions. The dried plates were inverted and incubated at 30°C for 6 d in a closed polyethylene bag to prevent dehydration of the medium. Diluted yeast cultures (0.1 mL) were also plated on basic and salt-containing YM Agars, dried, inverted and incubated at 30°C for 4 d, before the colonies were counted.

To enumerate the microorganisms growing in the canola mashes, both the basic media and media containing 18% NaCl were used, and 2.5 g of sodium propionate was added to each litre of medium. The yeast medium was then acidified to pH 4.5 with sterile 1 M HCl.

3.2 Preparation of Koji

3.2.1 Materials

(a) Defatted canola meal, ground and sifted through 40-mesh sieve.

(b) Wheat grains purchased from a local supplier.

(c) Alcalase 0.6 L (Novo Industri A/S, Enzyme Division, Novo Alle, Dagsvaerd, Denmark).

(d) Mould cultures: *Aspergillus oryzae* 14985 (NRRL 1989) and *Aspergillus soja* 16320 (ATCC, Rockville, MD).

3.2.2 Equipment

(a) Combi-Titrator 3D (Metrohm AG, Herisau, Switzerland). The combination of pH meter 512, Impulsomat E 473 and Multi-Dosigraph E 425 served as a regulating device to hold pH at a constant value while the canola meal was being digested by the enzyme. The

Dosigraph recorded the quantity of reagent that was required to keep the pH constant, thus permitting the progress of hydrolysis to be followed. The Multi-Dosigraph was connected to a double-walled, jacketed hydrolysis vessel with a multi-socket lid which had openings for an autoburette and pH electrode, and which was maintained at a constant temperature with a water bath. The hydrolysis substrate in the vessel was automatically titrated with 1 M NaOH to maintain a constant pH.

(b) Temperature Controlled Water Bath (Thermomix 1480, B. Braun, West Germany).

(c) Wine Press (Walker Desmond, Stockport, England), manually operated.

(d) U-V Hood (LabConCo Corp., Kansas City, MO).

3.2.3 Procedure

The temperature of the hydrolysis vessel was maintained at 69°C with the water bath. Defatted canola meal (50 g) was mixed with 210 mL of distilled water and 15.4 mL of alcalase 0.6L in the hydrolysis vessel and stirred continuously with a magnetic stirrer. Change of pH during the hydrolysis was monitored continuously with the Impulsomat pH meter, and the pH's deviating from the set pH of 9.0 were readjusted with 1 M NaOH, automatically fed through the autoburette into the hydrolysis vessel. The hydrolysis was terminated after 2 h and the volume of NaOH used was recorded by the Dosigraph.

After 2 h digestion, the pH of the digest was reduced to 5.5 with conc. HCl, and about 66% of the total hydrolysate in the digest was pressed out. Roasted and ground wheat (50 g) was added to the residue of the hydrolysed canola meal and mixed thoroughly. The final moisture content of the mixture was about 40-45%. The canola meal-wheat mixture was placed in a shallow sterile aluminum pan and aseptically inoculated with 0.1-0.2% per gram dry weight meal of a mixture of *A. oryzae* and *A. soja* prepared in section 3.1.1.3. The inoculated mash was incubated at 30°C for 72 h to form the koji. It was stirred after 20 and 40 h of incubation.

3.3 Moromi Fermentation

3.3.1 Moromi

Salt (NaCl) was added to 140 mL of enzyme hydrolysate, which had previously been pressed out of the canola-meal digest, to make an 18% w/v NaCl solution. This was added to the 72 h old mature koji which had been transferred into a sterile 500 mL wide-mouth erlenmeyer flask to form the salted mash (moromi). The pH of the moromi was adjusted to 6.5-7.0 with HCl.

3.3.2 Fermentation procedure

Three batches of moromi were prepared for the production of canola sauce (CS). They were identified as CS1, CS2 and CS3. The 'trained' microorganisms from section 3.1.3.1 were incubated in fresh 18% NaCl media prior to the preparation of the moromi. About 7 mL of 'trained' 7-day-old *P. halophilus* culture was inoculated into CS1 and CS3 contained in the sterile wide-mouth flasks, thoroughly stirred with sterilized spatulas, and plugged with sterile dispo plugs wrapped in aluminum foil. CS3 was also inoculated with about 7 mL each of 3-day-old cultures of *S. rouxii* and *T. versatilis*. CS2, the control, was not inoculated with any microorganism. All inoculations were done in a sterilized UV hood.

The moromi mashes were aseptically sampled before the inoculations and analysed for composition. The inoculated CS1 and CS3 and uninoculated CS2 were incubated at 30°C. The mashes were stirred and aseptically sampled every 2-4 d throughout the 31 d period of fermentation, for chemical analysis and viable counts of microorganisms present. The analyses included total soluble solids (TSS), total soluble nitrogen (TSN), glucose, sucrose, glycerol, lactic acid, ethanol, amino nitrogen (AN), pH and total titratable acidity (TTA) measurements.

3.4 Extraction

3.4.1 Materials and equipment

(a) Wine Press (Walker Desmond, Stockport, England), manually operated.

(b) Cheese cloth and glass sample bottles.

3.4.2 Procedure

The fermented mash was wrapped in layers of cheesecloth, placed in the wine press, and pressure was manually applied by means of a screw. The extract obtained was the raw canola sauce.

3.5 Refining

This process involved pasteurization and filtration of the sauce before bottling.

3.5.1 Equipment

(a) AMSCO Lab/Isothermal Autoclave (American Sterilizer Co., Erie, PA).

(b) 250 mL polycarbonate centrifuge bottles (Beckman Instr. Inc., Fullerton, CA).

(c) Preparative Centrifuge equipped with JA-14 rotor (Beckman Instr. Inc., Fullerton, CA).

(d) Whatman No. 1 filter pads, 12.5 cm (Fisher Scientific Co., Fair Lawn, NJ).

3.5.2 Procedure

The raw canola sauces contained in the sample bottles were loosely capped and placed in the isothermal autoclave, which had been set at 80°C. The samples were heated for 30 min. The pasteurized sauces were cooled slowly at room temperature to allow sedimentation of proteinaceous material. The cooled sauces were decanted into centrifuge bottles and centrifuged at 10,000 rpm for 30 min in the JA-14 rotor. The supernatants were then filtered in the sterile UV hood.

The manufacturing process for the production of the canola sauce is outlined in Figure 3.1.

3.6 Soluble Components of Sauce

3.6.1 Fractionation of canola sauce

3.6.1.1 Materials and Equipment

(a) Cation-exchange resin: Dowex 50 W-X8, 100-200 mesh in H^+ form (SERVA Feinbiochemica, Heidelberg, FRG).

(b) Anion-exchange resin: Amberlite IRA-400, 20-50 mesh in Cl^- form (Terochem Lab. Ltd., Edmonton, Alta.).

(c) Büchi-Rotavap-R (Foss Electric Canada Ltd., Cornwall, Ont.) with water bath maintained at 23°C and 30°C.

(d) Burettes; 4 M NH_4OH; 1 M NaOH; 1 M HCl.

3.6.1.2 Procedure for fractionation

A. Preparation of exchange columns

Cation-exchange column. A slurry of cation-exchange resin was packed to 15 cm height in a burette plugged at the bottom with glass wool. Distilled water acidified with 1 M HCl to pH 1 was slowly passed through the column to equilibrate the resin. The excess acid was washed off with distilled water until the effluent was neutral to pH paper. A layer of glass wool was also placed on top of the resin column.

Anion-exchange column. A slurry of the anion-exchange resin was packed into a burette to a height of 25 cm. The Cl^- form was converted to the OH^- form by slowly passing 1 M NaOH through the column. About twenty-bed-volumes of NaOH were used for the conversion, after which the excess NaOH was washed off with distilled water until the effluent was neutral to pH paper. The top of the resin bed was covered with glass wool to prevent disturbance of the resin layer.

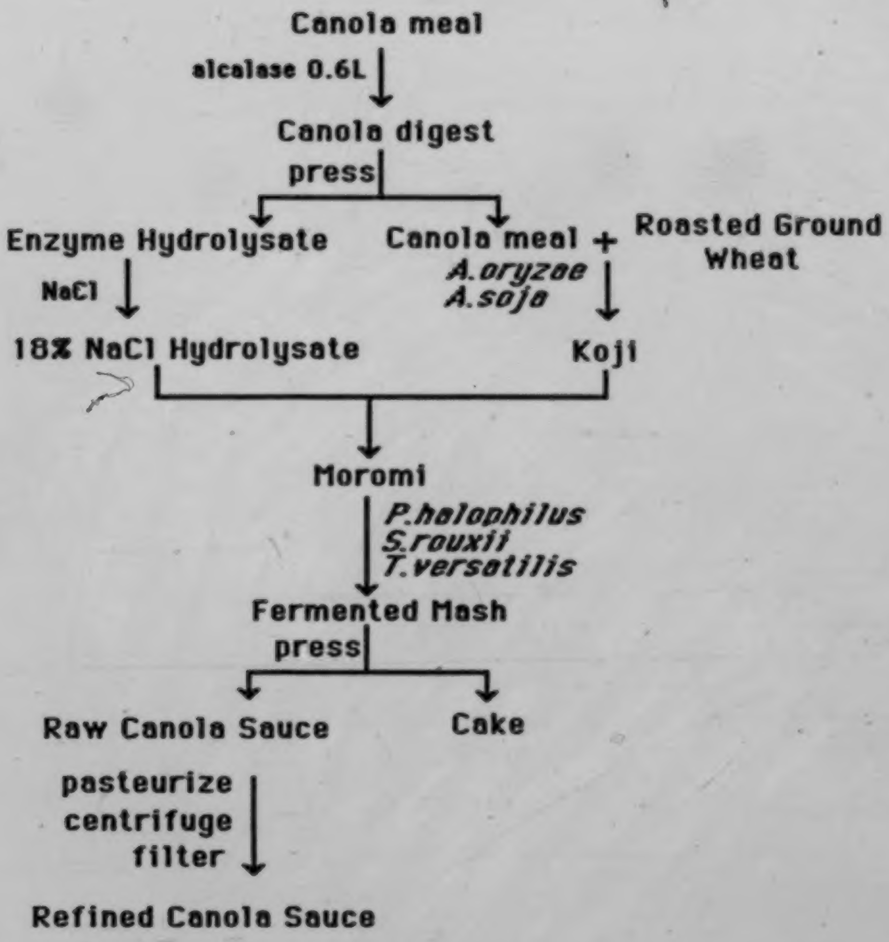

Figure 3.1 Outline of canola sauce production process

B. Fractionation

Two mL of canola sauce sample, pH adjusted to 1.5-2.0 with 1 M HCl, was gently poured down the cation-exchange column. The sample was allowed to penetrate the resin bed until it was level with the top layer of the resin. The column was washed with 40 mL of water and 40 mL of effluent was collected at a rate of 1 drop per sec. This 40 mL of effluent was further passed through the anion-exchange column and washed with 60 mL water. A total of 100 mL of effluent was collected as the neutral fraction.

The cation-exchange column was eluted with 110 mL of 4 M NH_4OH, slowly, and 110 mL of effluent was collected. This contained the nitrogenous compounds and amino acids.

The anion-exchange column was then washed with 200 mL of 1 M NaOH and 200 mL of effluent was collected. This fraction contained the organic acids, which were in the form of Na^+ salts.

C. Regeneration of exchange resins

Water was slowly passed through the cation-exchange column to wash off the NH_4OH completely, followed by acidified water, pH 1, to convert the counter-ion back to H^+. About 3-bed-volumes of the acidified water was used.

The anion-exchange column was similarly washed with water, followed by 1 M HCl to regenerate the Cl^- form of the resin.

D. Concentration of fractions

Ten mL of the NH_4OH fraction was concentrated by evaporation in the Rotavap at 30°C, and made up to 10 mL with distilled H_2O acidified to pH 2.2 with conc. HCl. This was used for the amino acid analysis.

The NaOH fraction containing the organic acids was also concentrated by evaporation in the Rotavap at 23°C, then diluted 10x with distilled water.

3.7 Compositional Analysis

3.7.1 Total soluble solids (TSS) measurement

One gram of mash, taken over the period of fermentation (every other day in the first week, then every 4 days thereafter until the fermentation was terminated on day 31) was pressed to extract the liquid. A drop of this extract was used for TSS measurement with an Abbe refractometer (Carl Zeiss, W. Germany).

3.7.2 pH measurement

The residue, after extracting the liquid from the samples taken, was used for the pH measurement. The method was adapted from A.O.A.C. 14.022 (1980). Two grams of residue was weighed into a clean, dry beaker and 20 mL of recently boiled water at 25°C was added. The mixture was shaken and stirred for 30 min to suspend the particles evenly. It was allowed to stand for 10 min, after which the supernatant was decanted and pH determined immediately with a Fisher Accumet Selective Ion Analyzer model 750.

3.7.3 Analysis of nitrogen-containing compounds

3.7.3.1 Quantitation of total soluble nitrogen (TSN)

TSN of the samples was determined on 0.3 mL of the extract, using a micro-Kjeldahl method adapted from A.O.A.C. 47.021 (1980).

3.7.3.2 Quantitation of amino-nitrogen (AN)

AN of the samples was determined on 2 mL of 1000x diluted extract with a method adapted from A.O.A.C. 10.179 (1980). A Beckman DU-8 spectrophotometer was used for absorbance measurement at 570 nm.

3.7.3.3 Quantitation of amino acids

A Beckman Amino Acid Analyzer model 121 MB (Beckman Instr., Inc., Palo Alto, CA) was used for the analysis of amino acids. The cation-exchange effluent was acidified with dilute HCl, pH 2.2, injected into sample storage coils, and placed on the turntable of the automatic sample injector (ASI).

A continuous stream of 3 buffer solutions was pumped consecutively through the resin column, after the sample had been injected at a point between the buffer pump and the resin column. This sample became a part of the eluent stream. The amino acid components present were separated on the column by the differences in their affinities for the resin. The column effluent was mixed with ninhydrin reagent, passed through a heated reaction coil for colour development, and then through a colorimeter for detection. The colour intensity of each amino acid was recorded on a chart recorder.

The operating instructions, as provided in the users manual for the Beckman Amino Acid Analyzer, model 121 MB, are as follows: column size 2.8 x 300 mm; resin AA-10; resin bed height 200 mm; buffer flow rate 10 mL/h; ninhydrin flow rate 5 mL/h; first buffer pH 3.28, 0.20 N Na^+; second buffer pH 3.90, 0.35 N Na^+; third buffer pH 4.95, 0.140 N Na^+; sample-diluting buffer pH 2.20; regenerating reagent NaOH, 0.20 N; column temperature, high 65°C; column temperature, low 50°C.

3.7.4 Analysis of acidic compounds

3.7.4.1 Quantitation of total titratable acidity

One mL of the pasteurized sauce was diluted 5x, decolorized with about 1 g of pre-prepared carbon decolorizer, and filtered through Whatman No. 1 paper. One mL of filtrate was titrated with 0.005 M NaOH, using phenolphthalein as indicator. The method was adapted from Onaga *et al.* (1957).

3.7.4.2 Quantitation of lactic acid

A 0.1 mL aliquot of a 100x diluted extract from the mashes, and also 0.1 mL each of raw and pasteurized sauces (100x diluted) were used to determine the amounts of lactic acid present. A UV-method, adapted from Methods of Enzymatic Food Analysis (Food Analysis, Boehringer Mannheim), involving test combinations, was used.

The principle involved in the determination is as follows:

(1) L-lactate + $NAD^+ \xrightleftharpoons{\text{L-LDH}}$ pyruvate + NADH + H^+

(2) pyruvate + L-glutamate $\xrightleftharpoons{\text{GPT}}$ L-alanine + α-oxoglutarate

where L-LDH = L-lactate dehydrogenase; GPT = glutamate-pyruvate transaminase.

The amount of NADH formed in reaction (1) is stoichiometric with the concentration of L-lactic acid; as such the increase in NADH is determined by means of absorbance at 340 nm. The general calculation formula is:

$$\text{Concentration (C, g/L)} = (V \times MW \times \Delta A)/\epsilon \times d \times v \times 1000$$

where: V = final volume of reaction mixture (mL) = 2.24; v = sample volume (mL) = 0.1; MW = molecular weight of lactic acid = 90.1; d = light path (cm) = 1; ϵ = adsorption coefficient of NADH at 340 nm (L $mmol^{-1}$ cm^{-1}) = 6.3.

Therefore:

$$C = (2.24 \times 90.1 \times \Delta A) / (6.3 \times 1 \times 0.1 \times 1000)$$

$$= 2.018 \times (\Delta A/6.3) \times F$$

giving g lactic acid / L sample solution, and where: ΔA = absorbance difference; F = dilution factor = 100.

3.7.4.3 Quantitation of organic acids using HPLC

Materials

Fisher Scientific: glacial acetic acid, 99.8%; oxalic acid, anhydrous; propionic acid, 99%; succinic acid, 99.6%.

Sigma Chem. Co.: citric acid, anhydrous; glycolic acid; lactic acid, 30% solution; pyroglutamic acid; pyruvic acid, Na^+ salt, crystalline.

Aldrich Chem. Co.: L-malic acid, 99%.

BDH Chemicals: formic acid, 98%.

0.008 N sulphuric acid.

Equipment

The HPLC system consisted of a Model 300 LC pump in combination with a Model 210 Guardian (Scientific Systems, Inc.) and a Spectro Monitor D variable wavelength detector with automatic zero (LDC/Milton Roy, Riviera Beach, FL). Results were recorded with an integrator (Varian Model 4270) and a recorder (Varian Associates Inc., CA). A 20 μL loop was attached to the pump and to the HPLC column for a constant 20 μL loading of the sample. The syringe for sample injection was from Rheodyne, Inc. (CA). A resin-based Aminex HPX-87H column was used both with and without a column heater. Both the column and the column heater were manufactured by Bio-Rad Laboratories (Mississauga, Ont.).

Procedure

A 0.008 N H_2SO_4 solution was employed as the eluent for the analysis, and the conditions used were: column temperatures, room temperature and 65°C; U-V detector monitored at 210 nm; U-V range 0.1; attenuation 32 and 64; chart speed 0.5 cm/min; mobile phase (eluent) flow rate 0.7 mL/min.

Solutions of the individual organic acids, as found in commercial soy sauce (Yokotsuka, 1986), were prepared and analysed in triplicate with the HPLC at both temperatures, and retention times and peak areas were recorded.

A calibration curve was made for the standard organic acids by plotting peak areas against the concentration of the acids.

The organic acid fraction, obtained from the anion-exchange column, was then analysed in a similar manner.

Retention times of the peaks were compared to those found for the standard organic acids to identify the individual acids present. The samples were then spiked with known organic acids to confirm the acid identifications. Individual acids present in the sample were quantified using the standard curve.

3.7.5 Analysis of sugars

3.7.5.1 Quantitation of glucose as reducing sugar and sucrose as non-reducing sugar

The analyses were performed on 0.1 mL of the 100x diluted samples. The glucose concentration was determined before and after the enzymatic hydrolysis, using the UV-method from Methods of Enzymatic Food Analysis (Food Analysis, Boehringer Mannheim). The principle for this method involved the determination of glucose before inversion reactions (1) and (2), and then the enzymatic inversion, reaction (3):

(1) Glucose + ATP $\xrightarrow{HK}$ G6P + ADP

(2) G6P + NADP$^+$ $\xrightarrow{G6P\text{-}DH}$ gluconate-6-phosphate + NADPH + H$^+$

The NADPH formed in the above reaction was stoichiometric with the amount of glucose and was measured by the increase in absorbance at 340 nm.

(3) Sucrose + H_2O $\xrightarrow{\beta\text{-fructosidase}}$ glucose + fructose

The difference between the glucose before and after enzymatic inversion was calculated as the sucrose content. Absorbance was measured at 340 nm. HK = hexokinase. G6P-DH = glucose-6-phosphate dehydrogenase.

The general calculation formula is:

$$\text{Concentration } (C, g/L) = (V \times MW \times \Delta A)/\epsilon \times d \times V \times 1000$$

For sucrose concentration:

$$C = (3.02 \times 342.30 \times \Delta A_{sucr}) / \epsilon \times 1 \times 0.1 \times 1000$$

$$= 10.34 \times (\Delta A_{sucr}/6.3) \times F$$

giving g sucrose/L sample solution; where: $\Delta A_{sucr} = \Delta A_{total\ gluc} - \Delta A_{gluc}$; F = dilution factor (100).

For glucose concentration:

$$C = (3.02 \times 180.16 \times \Delta A_{gluc}) / 6.3 \times 1 \times 0.1 \times 1000$$

$$= 5.441 \times (\Delta A_{gluc}/6.3) \times F$$

giving g glucose/L sample solution.

3.7.6 Analysis of alcohols

3.7.6.1 Quantitation of ethanol

Ethanol determination was done on 0.1 mL of the 100x diluted sample extract. The principle required that the NADH produced (which was stoichiometric with half the amount of ethanol present) be measured at 340 nm. This procedure was adapted from Methods of Enzymatic Food Analysis (Food Analysis, Boehringer Mannheim).

Reactions:

(1) Ethanol + $NAD^+ \xrightleftharpoons{ADH}$ acetaldehyde + NADH + H^+

(2) Acetaldehyde + NAD^+ + $H_2O \xrightarrow{AL\text{-}DH}$ acetic acid + NADH + H^+

where: ADH = alcohol dehydrogenase; AL-DH = aldehyde dehydrogenase.

The general calculation formula is:

$$\text{Concentration (C, g/L)} = (V \times MW \times \Delta A)/\epsilon \times d \times V \times 2 \times 1000$$

yielding:

$$C = (3.15 \times 46.07 \times \Delta A) / 6.3 \times 1 \times 0.1 \times 2 \times 1000$$

$$= 0.7256 \times (\Delta A/6.3) \times F$$

giving g ethanol/L sample solution, and where: ΔA = absorbance difference in sample; F = dilution factor (100).

3.7.6.2 Quantitation of glycerol as polyalcohol

The UV-method adapted from Methods of Enzymatic Food Analysis (Food Analysis, Boehringer Mannheim) was used in the determination of glycerol. This involved catalysed reactions which consumed NADH. The NADH used up was stoichiometric with the amount of glycerol present, and this was determined by measuring absorbance at 340 nm.

(1) Glycerol + ATP $\xrightarrow{GK}$ glycerol-3-phosphate + ADP

(2) ADP + PEP $\xrightarrow{PK}$ ATP + pyruvate

(3) Pyruvate + NADH $+H^+ \xrightarrow{LDH}$ lactate + NAD^+

where: GK = glycerokinase; PK = pyruvate kinase; LDH = lactate dehydrogenase.

The general calculation formula is:

$$\text{Concentration (C, g/L)} = (V \times MW \times \Delta A)/\epsilon \times d \times V \times 1000$$

yielding:

$$C = (3.02 \times 92.1 \times \Delta A) / 6.3 \times 1 \times 0.1 \times 1000$$

$$= 2.781 \times (\Delta A/6.3) \times F$$

giving g glycerol/L sample solution, and where: ΔA = absorbance difference of sample; F = dilution factor (100).

3.7.6.3 Quantitation of phenylethanol

Reagents

Phenylethanol (phenethyl alcohol or PEA) standard solutions:

(1) 1% stock solution (10,000 ppm) prepared in distilled water; reagent from Eastman Kodak Co. (Rochester, NY).

(2) Working solutions: 1, 5, 10, 25, 50 ppm (by diluting stock solution with distilled water).

Equipment

A Varian Model 3700 gas chromatograph equipped with hydrogen flame ionization detector and a Tenax-GC column (Applied Science Labs., Inc., PA) was used. The chromatograms were recorded on a chart recorder (Cole-Palmer Instr. Co.).

Procedure

The separation of phenylethanol was made under the following operating conditions: column packing, Tenax-GC, 60-80 mesh; carrier gas, helium, 41.5 p.s.i. inlet pressure, 25 mL/min flow rate; detector, flame ionization; hydrogen, 30 p.s.i.; air, 60 p.s.i.; column temperature, 210°C; injector temperature, 250°C; detector temperature, 270°C.

Four μL of each working solution was injected several times into the gas chromatograph. The PEA peaks eluted after about 4 min and the peak heights were measured in mm.

A plot of average peak height against concentration in ppm of the working solutions was made to obtain a standard curve (Figure 3.2). Four μL of 20x diluted pasteurized canola sauces and Kikkoman sauce were also injected several times, and peak heights measured. Spiking with known amounts of 50 ppm working solution to sauce samples was done to confirm the identification of PEA in the sauces. PEA in the sauces was quantified using the standard curve. This procedure was a slight modification of that described by Kahn and Conner (1972).

3.7.7 Sodium chloride determination

A Corning pH/ion meter 150 (Corning Science Products, Essex, England), attached to reference and sensing electrodes, was used to measure the electrode potential (mV) for NaCl determination. The amount of NaCl present was expressed in terms of % chloride.

Twenty-five mL of standard solutions of NaCl and 25 mL of sample solutions diluted 100x were adjusted with 0.5 mL of ISA (ionic strength adjustor, a 5 M $NaNO_3$ solution) before mV readings were taken. A standard curve (Figure 3.3) was plotted, showing mV of standard NaCl solutions against molarity. The chloride concentration in samples was determined from the curve.

3.7.8 Moisture determination

An Automatic Volatility Computer Model AVC-MP (CEM Corp, Indian Trail, NC) was used for the determination of moisture content in the samples. The principle involved % weight loss due to drying by exposure to microwave radiation. The water present was selectively heated and removed through vaporization, using 0.5 g of sample and recording moisture content automatically with the instrument.

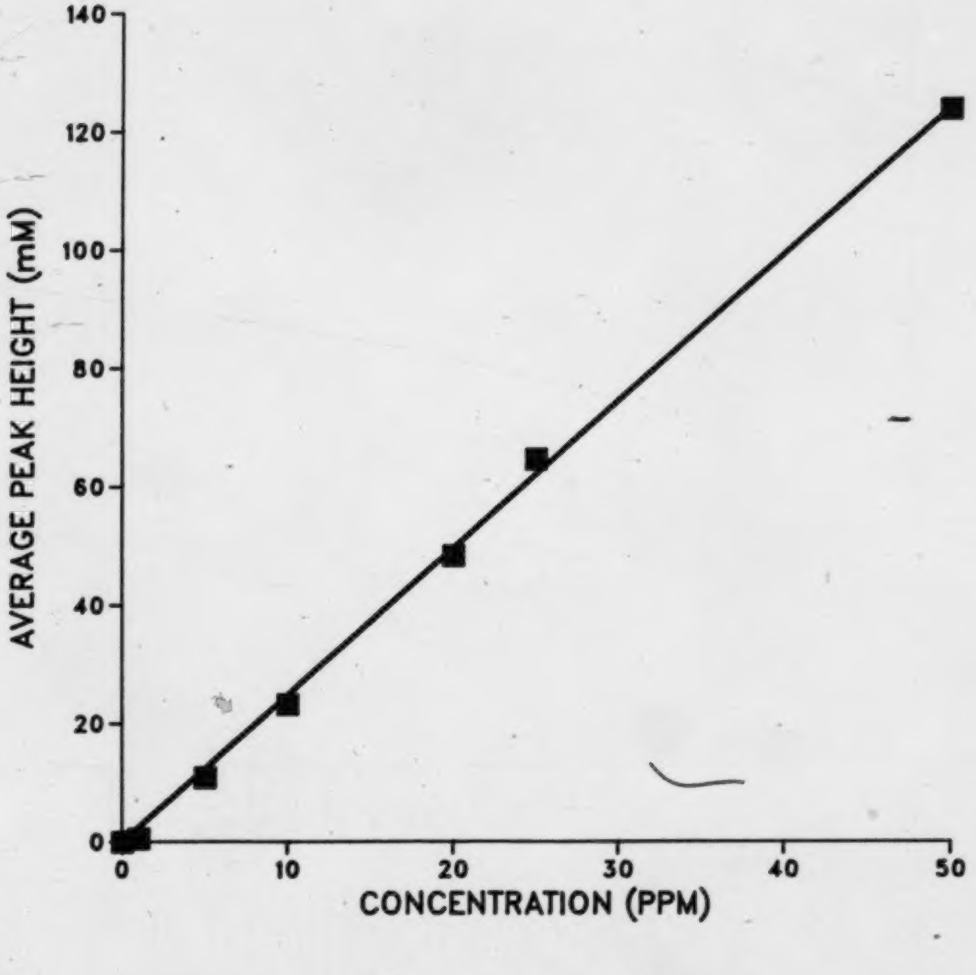

Figure 3.2 Standard curve for determination of phenylethanol concentration (average of four determinations). Equation of line:

Height (mm) = -1.09 + 2.51 x concentration (ppm)

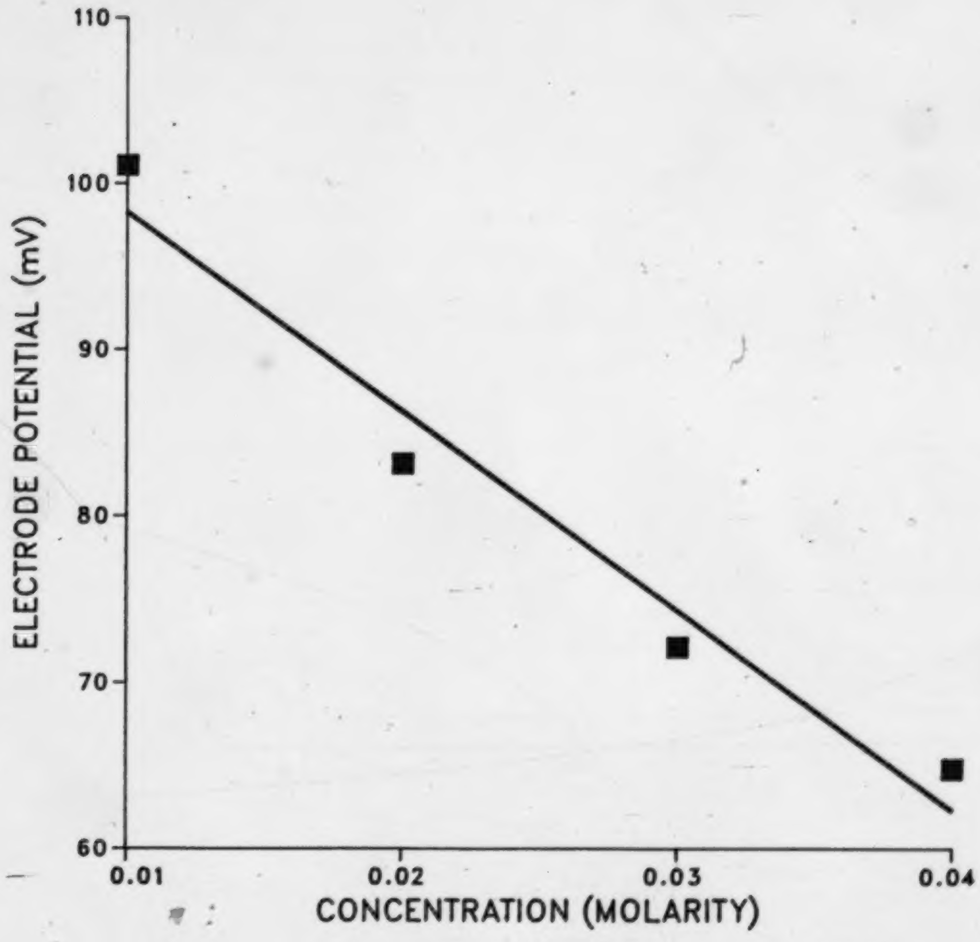

Figure 3.3 Standard curve for determination of sodium chloride concentration (average of three determinations). Equation of line:

Electrode potential (mV) = 110.3 - 1199.5 x Molarity

3.8 Colour Measurement

A Hunter L,a,b Colorimeter model D25L-2 and detector D25 Optical Head (Hunterlab, Fairfax, VA) was used for the colour measurement of sauces. The different coloured ceramic tiles were used to standardize the instrument before sample readings were recorded. Aliquots of 20 mL of each sample placed in petri-dishes were used for the measurements.

3.9 Sensory Evaluation Tests

Two sensory evaluation sessions, involving 7 panelists from the Department of Food Science, University of Alberta, were conducted.

The 3 canola sauces produced in the experiment were compared to Kikkoman sauce purchased on the local market. A 9-point hedonic scale was used, where a score of 9 indicated an extreme preference or liking for the samples, and 1 indicated an extreme dislike or the least preferred.

The attributes studied were colour, flavour, aroma and the overall acceptability of the sauces.

Statistical evaluation, involving analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT), was performed on the results obtained for the sensory evaluation.

4. RESULTS AND DISCUSSION

4.1 Training of Microorganisms

To obtain the inocula for the canola mash stage of the sauce fermentation, it was essential to 'train' the microorganisms to adapt to and grow in a high saline environment similar to that found during brine fermentation. As reported by Yong and Wood (1976), 'untrained' cultures did not grow when inoculated directly into media of high salt content.

It was observed during this experiment that each transfer of microorganisms into media with increasing salt content resulted in a lag period of growth. Also observed was the fact that, even when the microorganisms were transferred directly into media with 18% w/v NaCl media, growth occurred after a lag period. Therefore, transfers through media with increasing salt content were deemed unnecessary and, subsequently, cultures were inoculated directly into media containing 18% w/v NaCl, followed by a series of subculturing into the same high salt media.

As observed by Yong and Wood (1974), viable cell counts decreased on subculturing. Figure 4.1 shows that *S. rouxii* and *T. versatilis* grew to their maximum viable cell counts of 7×10^7 and 1.5×10^8 CFU/mL, respectively after 3 days of incubation, which corresponded to optical density (OD) values of 1.50 and 1.78 (Figure 4.2). However, further incubation resulted in a slight decrease in viable cell counts. *P. halophilus*, on the other hand, exhibited a much longer lag phase beginning with a slight decrease in viable cell count within the first three days (Figure 4.1) and did not reach the maximum count of 3×10^7 CFU/mL, corresponding to an OD of 0.33, until the ninth day.

It is interesting to note that the increase in the OD values of the inocula corresponded well with the increase in viable cell counts of the yeasts initially, until the counts reached a maximum. However, the OD continued to rise even when the cell counts started to decrease. This suggested that, as the cells continued to reproduce, a considerable number of the cells were killed. The OD measured the cell mass in the broth, while only live cells were accounted for in the viable cell count.

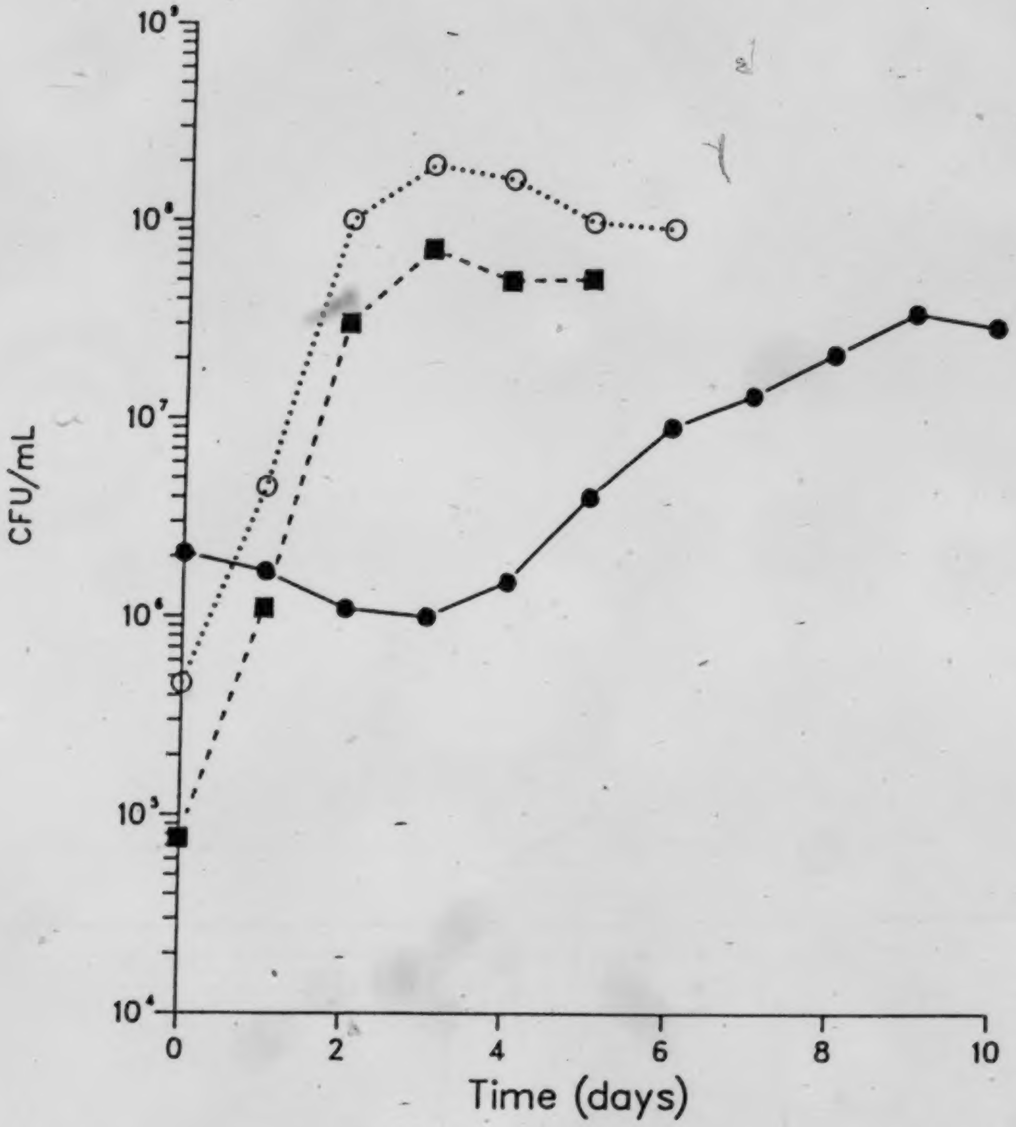

Figure 4.1 Growth of 'trained' microorganisms (average of duplicate plate counts). (●) *P. halophilus*; (■) *S. rouxii*; (○) *T. versatilis*.

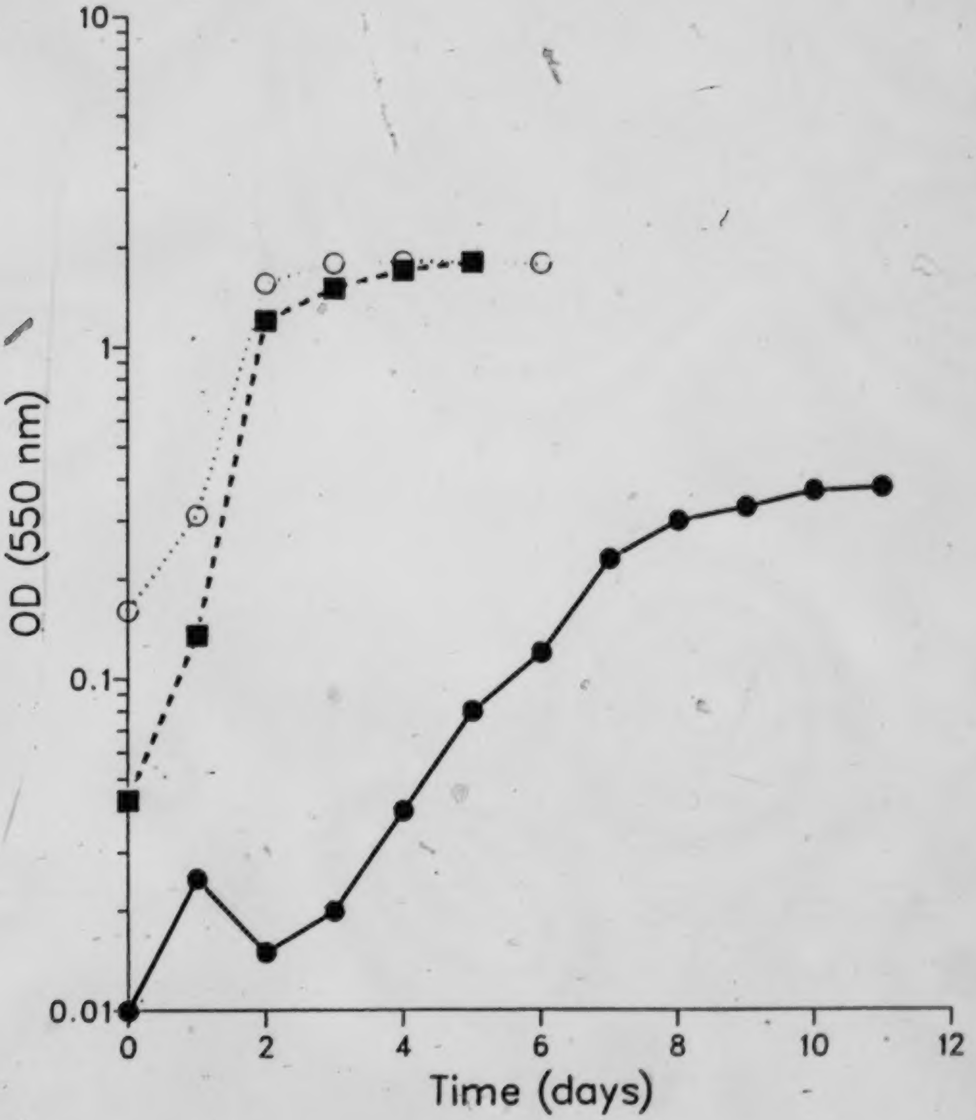

Figure 4.2 Optical density (OD) measurements of growth of 'trained' microorganisms at 550 nm (average of two determinations). (●) P. halophilus; (■) *S. rouxii*; (○) *T. versatilis*.

The culturing method described by Yong and Wood (1976) was modified by reducing the incubation temperature from 40°C to 30°C, since this temperature has been used successfully by Japanese workers for both microbial incubation and sauce fermentation.

Yong and Wood (1976) established the succession of microbial growth in soy mash and noted that lactic acid bacteria initiated the fermentation process at pH 6.5-7.0. As well, their 'trained' *Lactobacillus delbrueckii* was properly conditioned after 7 d of incubation. Thus, 'trained' *P. halophilus* was cultured statically at pH 7 in Sodium Acetate Medium 1 (SAM 1), containing 18% salt, for 7 d before being inoculated into the canola mash with similar environmental conditions. Both species of 'trained' yeasts were conditioned by incubating them for 3 d in the 18% NaCl-YM medium before inoculation into moromi mash. The pH of the NaCl-YM medium was adjusted to 4.5 with sterile 1 M HCl, as did Yong and Wood (1976). However, Yong *et al.* (1978) proposed that, for greater growth, acidification of yeast growth medium should be done with lactic acid. However, since lactic acid was to be measured as a metabolite produced solely by *P. halophilus*, it was important that no lactic acid reagent be used for acidification. As such, HCl was used to maintain pH at 4.5 in the YM medium.

4.2 Koji Preparation

Ma (1985), using response surface methodology, obtained optimum conditions for the hydrolysis of canola meal by alcalase 0.6L. At a temperature of 69°C, pH 9, enzyme/substrate (E/M) ratio 0.31 (v/w), and meal/solvent (M/S) ratio of 1:5, the maximum yield of total soluble nitrogen (TSN) after 2 h was 0.848%. Applying a similar procedure in this experiment, the TSN yield for the canola meal hydrolysis was 0.917%. The higher TSN value here could be attributed to the final M/S ratio obtained after the required 2 h reaction period. With the automatic addition of 1 M NaOH by the pH-stat to maintain pH 9, the resultant M/S ratio was approximately 1:6.3. This was closer to the optimum ratio of 1:10 found by Ma (1985) than 1:5 which she actually used in her experiments. Ma (1985) had to use the M/S ratio of 1:5 to obtain a more concentrated hydrolysate to produce a sauce with a concentration comparable to soy sauce. Also, experiments had shown that the increase in TSN yield leveled off from the ratio of 1:5 and higher. In this experiment, the M/S ratio of 1:6.3 was maintained throughout.

The pH of hydrolysed canola meal was adjusted to 5.5 before about 66% of the liquid was pressed out for latter use. The residue was mixed with roasted ground wheat, which reduced its moisture content to 40-45%. This level of moisture content, together with pH of 5.5, was necessary for the growth of the *Aspergillus* moulds. The canola-wheat mixture, inoculated with *A. oryzae* and *A. soja*, formed the koji, which was incubated at 30°C for 72 h. Stirring of the koji after 20 and 40 h (Yong and Wood, 1976) was necessary to cool the material. Yokotsuka (1985) reported that the koji incubated for 72 h sometimes reached temperatures of over 40°C, which harmed the mould. Thus, stirring at intervals kept the koji fairly open to penetration of air, ensured an even distribution of water through the koji, and prevented execssive drying and early sporulation of the mould, and also over-heating of the koji mass (Wood, 1982).

After 72 h, the mature koji had a yellowish-green appearance, a slight ammoniacal smell and a reduced moisture content of about 30-35%. It was then given a final stir and mixed with 18% salt solution. The salt solution was made by dissolving salt in the enzyme hydrolysate which had been pressed out of the hydrolysed canola meal prior to the addition of the ground wheat. The salted enzyme hydrolysate then served as the immediate source of nutrients for the fermentation. On commercial scale, Yokotsuka (1985) reported that brine solution in ratio of 1.2-1.3:1 of raw materials was often used. Yong and Wood (1976), on the other hand, used a ratio of 1:2 of raw materials to brine solution for their experimental soy sauce production, and reported good quality product. Ma (1985), using a ratio of about 1:1.4 of raw materials to 18% NaCl-enzyme hydrolysate, was able to produce an acceptable sauce from canola meal. The ratio of 1:1.4 was used in this experiment so as to obtain a sauce similar in consistency to that produced by Ma (1985). By mixing the salted enzyme-hydrolysate with the matured koji, moromi was formed and this served as the substrate for the brine fermentation.

4.3 Brine Fermentation

In the natural fermentation process, the pH of soy mash at the start of brine fermentation is usually between 6.5 and 7.0. This, together with the available nutrients from the breakdown of proteins and carbohydrates from the raw materials, supported the growth of lactic acid bacteria and yeasts. Because fermentation time of canola sauce was shortened to 4-5 wk, the mash was deliberately inoculated with lactic acid bacteria and yeast cultures to improve ~~sauce quality.~~

Yong and Wood (1976) reported that lactic fermentation is followed by yeast fermentation when pH of mash drops below 5. To confirm this hypothesis, canola mashes CS1 and CS3 were inoculated with 'trained' *P. halophilus*, *S. rouxii* and *T. versatilis*, the former sequentially and the latter simultaneously. CS2 was used as a control and, therefore, was not inoculated with any of the microorganisms. Thus, in CS1, after *P. halophilus* had decreased the pH from 7 to about 5, *S. rouxii* and *T. versatilis* were inoculated into the mash. CS3, on the other hand, was inoculated with the three 'trained' microorganisms at the start of the brine fermentation.

To eliminate the possibility of contamination during the fermentation, the mashes were kept in sterile containers stoppered with sterile dispo plugs wrapped in aluminum foil, and incubated at 30°C for 31 d.

4.3.1 Microorganisms during moromi fermentation

4.3.1.1 Growth of microorganisms in canola mash

To prevent mould spores present in moromi mash from germinating and therefore overgrowing the plates used for enumerating bacteria and yeast cells, sodium propionate was added to the media, as suggested by Yong and Wood (1976). It had been observed from a number of experiments that adding NaCl to the basic medium, containing 0.25% sodium propionate, prevented mould growth. Yong and Wood (1976) used 5% NaCl/L of enumerating medium, while other studies have used as much as 18%. When salt was added to the enumerating medium to make 18% NaCl medium, dehydration of the medium was observed. Therefore, the inoculated plates were placed in polyethylene bags before incubation to minimize the dehydration. However, if NaCl was not added to the plating medium, the plates had to be incubated in an anaerobic jar to prevent mold growth when *P. halophilus* was enumerated. It was also observed that, when the mash samples were plated on medium containing 18% NaCl, the viable cell counts were greater than when plated on basic medium.

The growth of the organisms inoculated into the canola mashes is shown in Figures 4.3 and 4.4. In the 'stepwise' inoculated mash, CS1 (Figure 4.3), when *P. halophilus* was the only microorganism in the initial stages of fermentation, its viable count was greater compared to that in CS3, the 'all-in' inoculated mash, where yeasts were also present (Figure 4.4). This could be due to a possible competition for nutrients between the bacterium and the yeasts in CS3, thus neither grow to their full potential as in CS1. It has also been reported (Yokotsuka, 1986) that the salty conditions, the initial high pH and the presence of the lactic bacteria inhibited growth of yeasts. It was thus not surprising to notice a decrease in viable counts of the yeasts in CS3 (Figure 4.4) during the first week of brine fermentation.

The growth of *P. halophilus* resulted in more lactic acid being produced in CS1 than in CS3 (Tables 4.4 and 4.5) The lactic acid produced accelerated the pH drop in the mashes (Figure 4.6), thereby giving rise to a slightly lower pH in CS1. Although studies have indicated that the acidic and salty conditions, as well as the metabolites produced by the lactic bacteria, killed the bacteria by the end of the fermentation (Yong and Wood, 1976; Fukushima, 1985; Yokotsuka, 1986), the results from this experiment indicated that some of the bacteria survived but did not grow. It is possible that extending the fermentation period would result in a lower viable count.

Since the CS1 had initially been inoculated with *P. halophilus*, its growth rapidly reduced the initial pH to about 5 within 4 d. Therefore, when yeasts were subsequently inoculated, they grew rapidly (Figure 4.3). There was no period of inhibition as was observed in CS3 (Figure 4.4). This difference in growth of the yeasts (CS1 and CS3) was due to the fact that *S. rouxii* and *T. versatilis* in 18% salt medium grow best when pH is between 4.5 and 5.0 (Osaki *et al.*, 1985).

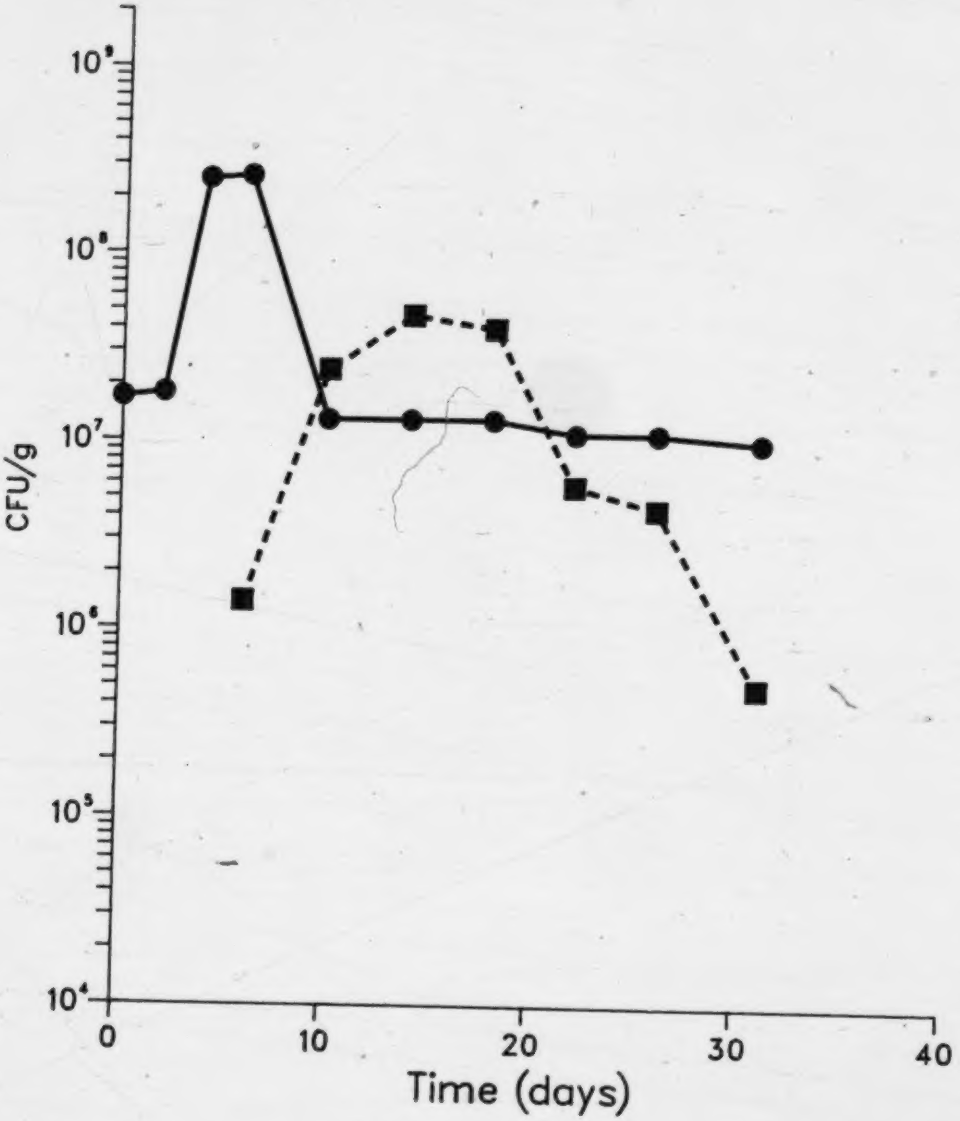

Figure 4.3 Viable counts of microorganisms: 'stepwise' inoculation (average of duplicate plate counts).

(●) *P. halophilus* ; (■) total yeast count.

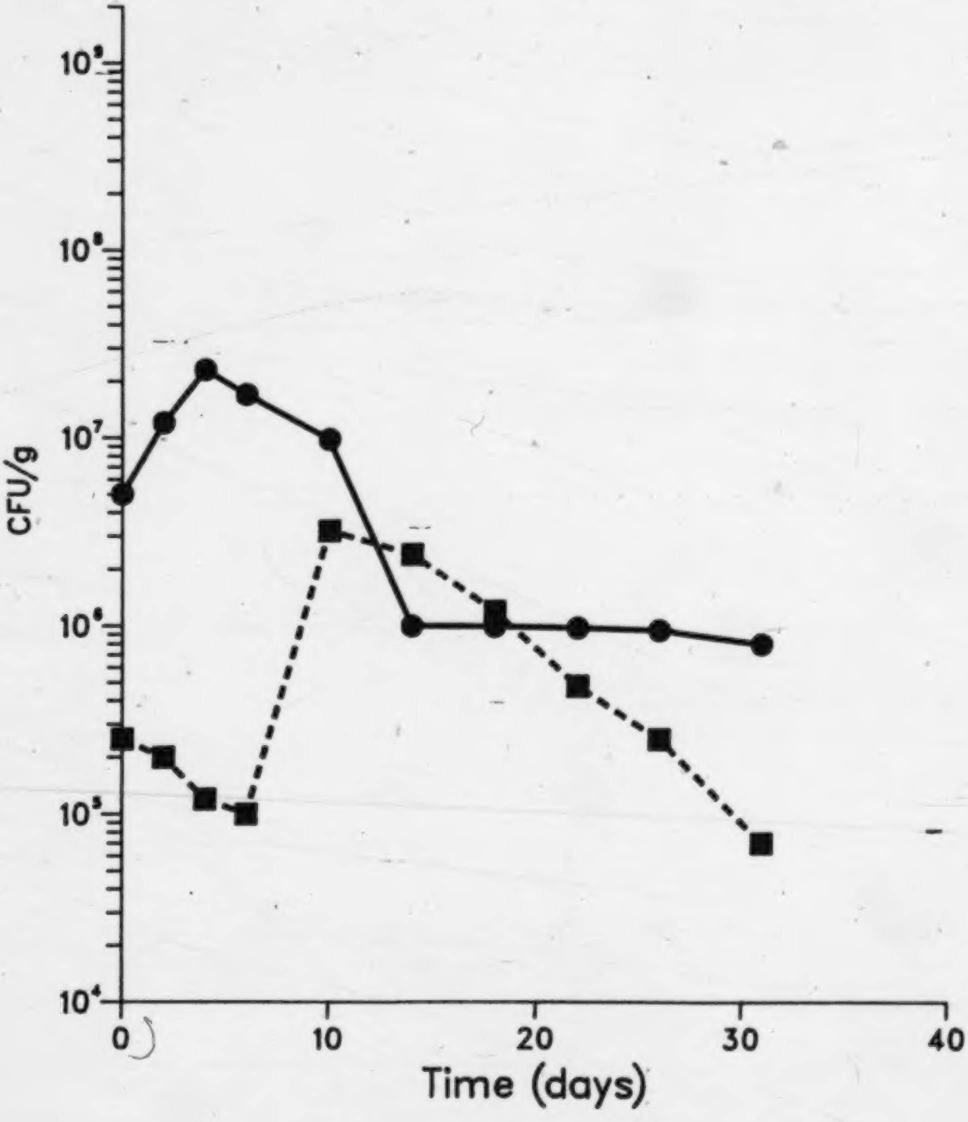

Figure 4.4 Viable counts of microorganisms: 'all-in' inoculation (average of duplicate plate counts).

(●) *P. halophilus* ; (■) total yeast count.

The curves shown in Figures 4.3 and 4.4 do not represent growth curves of the individual yeasts but a combined growth of both *S. rouxii* and *T. versatilis*. Studies have shown, however, that *S. rouxii* initiated the alcoholic fermentation and then lost most of its activity as the conditions in the mash changed. These conditions then favoured *T. versatilis*, which grew in more anaerobic conditions and even utilized nitrogen for growth (Ho *et al.*, 1984). Hence, *T. versatilis* became more active in the latter stage of fermentation.

4.4 Compositional Analyses

4.4.1 Total soluble solids (TSS) and pH changes during mash fermentation

As a result of degradation of components present in the mash, the soluble solids content increased from the beginning of the fermentation until day 31, when the fermentation was terminated (Figure 4.5). Both CS1 and CS3 started with a lower TSS value than CS2 due to the dilution of the mashes by inocula. Yet, within 4 d, the total increases in %TSS in the inoculated mashes exceeded that in the uninoculated mash. Obviously, the presence of the microorganisms caused faster degradation of the substrate. It is interesting to note that, although CS2 was not inoculated, the TSS content increased. In effect, degradation of the substrate was not only due to the microorganisms, but also to other factors, e.g. enzymes carried over from the koji, which are not affected by high salinity.

CS2 was tested at the start and at the end of fermentation for possible contamination with the inocula, but the results were negative. It was possible that contamination by adventitious microorganisms during incubation of koji occurred, as incubation was not done under sterile conditions. This implies that any contaminating microorganism involved must be other than *P. halophilus*, *S. rouxii* or *T. versatilis*.

There were two distinct rises of TSS in CS1 and CS3 within the first 2 weeks of fermentation. In addition, a slight rise was observed before the fermentation was terminated (Figure 4.5). A similar observation was reported by Ooraikul *et al.* (1980) in their conventional fermentation of canola mash. They attributed the first rise in TSS to acid

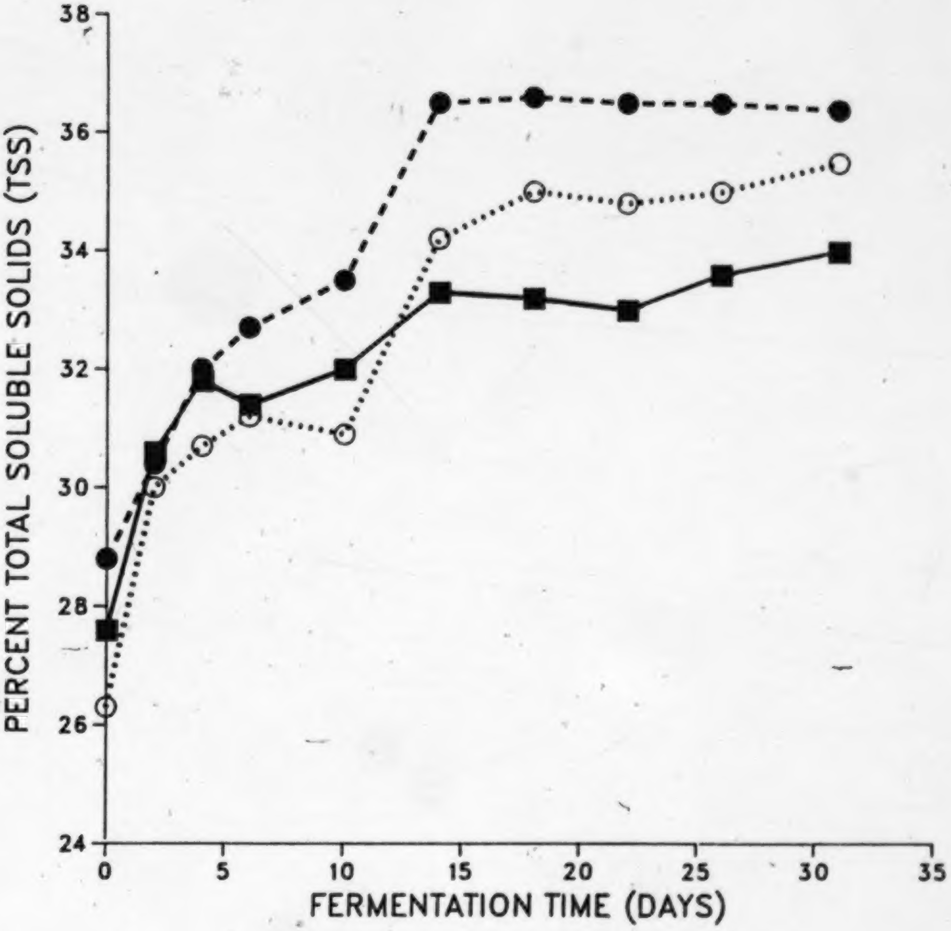

Figure 4.5 Changes in total soluble solids in canola mashes (average of two determinations).

(■) CS1: 'stepwise' inoculation of mash

(●) CS2: uninoculated mash (control)

(○) CS3: 'all-in' inoculation of mash

fermentation, where carbohydrates in the substrates were converted to sugar and organic acids, and proteins to amino acids. The second rise was attributed to alcoholic fermentation as well as sugar breakdown.

Yong and Wood (1976) also reported a two-step fermentation in their experimental soy sauce production. The first step involved lactic acid bacteria, followed by yeast growth in the second step. Ma (1985) reported that, although two rises were observed in one of her experiments, it was concluded that the second rise could not have been due to yeast growth, since in 18% salt medium yeast growth could occur only when pH is between 4.5 and 5.0 (Yong and Wood, 1976; Onishi, 1963). Hence, the second rise observed by Ma (1985) could be a result of some fluctuations in growth of other microorganisms.

The first rise in CS1 and CS3 corresponded with sharp decreases in pH (Figure 4.6). The pH in CS2 also dropped as the fermentation progressed. *P. halophilus* metabolized the sugar substrates predominantly to lactic acid, which accelerated the pH drop as well as increased TSS in CS1. The second rise occurred after the addition of the yeast inoculum. At pH of about 5, the added yeasts converted larger sugars to simple sugars, then to alcohol and volatile compounds, which increased TSS in CS1 further. Like CS1, the initial rise in TSS of CS3 was due to the growth and production of metabolites by *P. halophilus*. In addition, the yeasts might also metabolize the substrates at pH above 5. This is possible as Yong *et al.* (1980) reported that *S. rouxii* grown at pH 4.5 and inoculated into the reaction medium at pH 7, metabolized glucose into acids as well as ethanol and carbon dioxide. The second rise in CS3 occurred in a similar manner to CS1, i.e. after the pH had dropped to about 5. At this point the yeast activity was increased, resulting in a more vigorous growth, hence production of more metabolites. *T. versatilis* is known to dominate the latter part of fermentation, while *S. rouxii* activity is reduced due to the presence of all the metabolites. With reduced pH and the presence of these metabolites, *P. halophilus* activity is also reduced. Only *T. versatilis* is able to grow, which may have caused the slight TSS rise towards the end of fermentation in CS1 and CS3 (Figure 4.5). *T. versatilis* grows and produces other alcoholic and phenolic compounds known to be present in soy sauce.

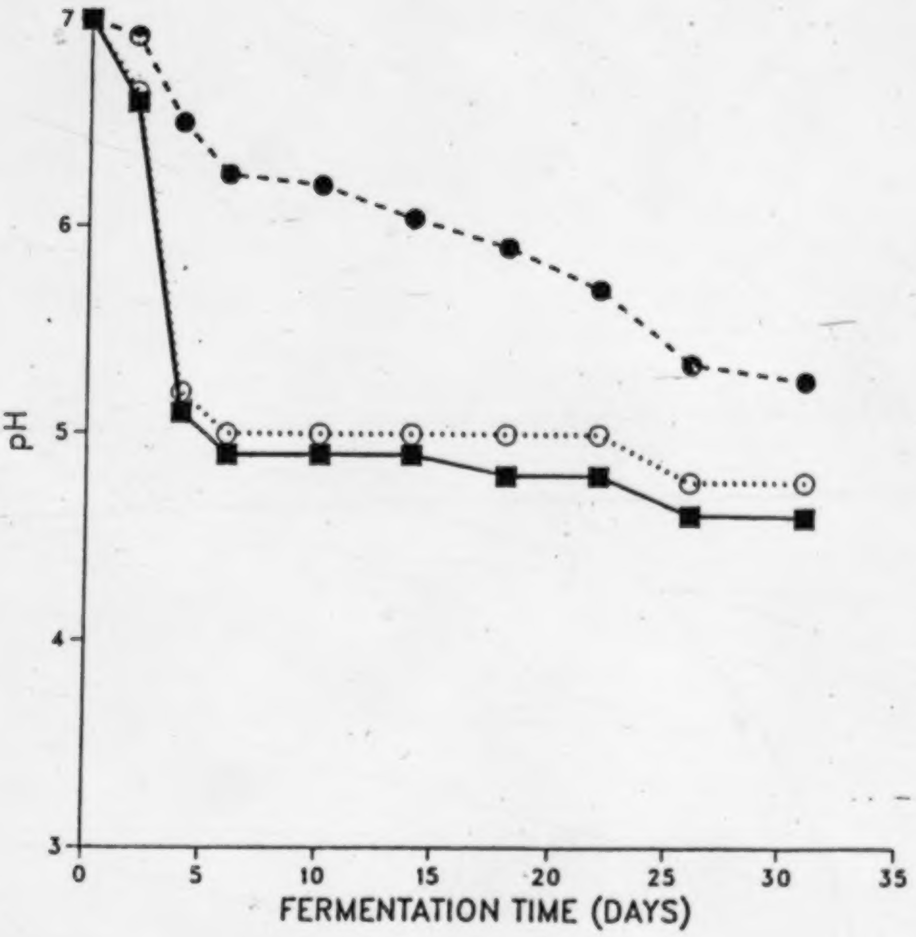

Figure 4.6 Changes in pH in canola mashes (average of two determinations).

(■) CS1: 'stepwise' inoculation of mash

(●) CS2: uninoculated mash (control)

(○) CS3: 'all-in' inoculation of mash

TSS in CS2 increased steadily from the beginning until it reached a plateau on day 15 of fermentation without any distinct breaks. With pH (Figure 4.6) above 5, and without any inoculation, there was no initiation of yeast growth, and the increase in TSS could only be attributed to the indigenous enzymes and/or microbial contaminants carried over from the koji to the moromi stage. Considering the difference in the metabolites in CS1 and CS3 (Tables 4.1-4.5) and the amount of residual sugars in CS3 (Tables 4.6 and 4.7), it was not surprising that CS3 contained more TSS than CS1, by the end of the fermentation. Although the *P. halophilus* inoculum was the same in both CS1 and CS3, the final pH was lower in CS1, i.e. 4.6 as compared to 4.8 in CS3. The difference is due to the fact that from the beginning of fermentation until day 4, only *P. halophilus* was present in CS1 producing lactic acid without any inhibition, unlike CS3 with all three microorganisms. The competition in growth created by the presence of the yeasts in CS3 suppressed the amount of acid produced by *P. halophilus*. Therefore, the pH was slightly higher in CS3, after the first 4 d, than in CS1. This slight pH difference was maintained throughout the fermentation (Figure 4.6)

Although CS2 was not inoculated, its pH also decreased. Unlike the koji for CS2, Yong and Wood (1976) produced their koji under strict aseptic conditions. Yet, they also observed a decrease in pH of the fermenting mash which had not been inoculated. Though there is reason to believe that CS2 could have been contaminated during the koji making stage and, therefore, the contaminants may have caused the pH to decrease, literature maintains that, under the conditions prevailing in the mash during sauce fermentation, no wild microorganism from the koji stage could survive. Those which do, e.g. *Bacillus subtilis*, remain as spores and do not take part in fermentation.

Although Yong and Wood (1976) searched for plausible reasons for this occurrence, all explanations eluded them. This observation, therefore, implies that whether or not koji is produced aseptically and the moromi made without inoculating lactic acid bacteria, its pH will decrease by the time fermentation ends. In effect, inoculating mash with lactic acid bacteria serves to accelerate the pH drop so that yeast fermentation can begin. This also indicates that canola sauce can be produced as successfully by inoculating the mash with cultured yeasts, after the pH of the mash has dropped by itself to about 5, when yeast growth is possible. However, this would require a much longer fermentation time.

4.4.2 Nitrogen-containing compounds

4.4.2.1 Changes in total soluble nitrogen (TSN) and amino nitrogen (AN) during mash fermentation

Yokotsuka (1986) cautioned that the use of too much lactic starter decreased protein digestibility. In this experiment, protein digestibility and, therefore, the final TSN of about 1.2%, was less than the value usually quoted in the literature, i.e. 1.5-1.8%. It was initially thought that perhaps the inoculum of *P. halophilus* used was too large; yet the viable counts of the bacteria on day zero (10^6-10^7 CFU/g) were comparable to the counts used by Yong and Wood (1976), i.e. 6.8 x 10^6 CFU/g, at the start of their fermentation. On the other hand, the commercial Kikkoman sauce used for comparison in this experiment had a lower %TSN value of about 1.33, when compared to values in the literature (Table 4.2). Although 1.33% is greater than the 1.2% observed in this experiment, Yokotsuka (1981) made the observation that %TSN in soy sauce varied from brand to brand and from year to year, even of the same brand. Therefore, the %TSN reported in this canola sauce could still be favourably compared with that of the commercial sauce. In addition to this observation, Ma (1985) also reported %TSN values of 1.12-1.34%, which were slightly lower than that of the commercial soy sauce used for comparison (Table 2.10).

During the 31 d of fermentation, CS3 showed the greatest increase in %TSN (0.507%), followed by CS1 (0.46%) and then CS2 (0.26%) (Table 4.1).

Yong and Wood (1977b) alluded to the fact that the increase in TSN in their sauces inoculated with yeast alone could have been the result of some protection and/or promotion of the mould enzymes by the yeast. Or, perhaps, that the shift of pH from the initial neutral value to 4.5 could have benefited the mould's acid proteinase such that more TSN was produced. Comparing the overall increase of TSN in CS1 and CS3 to CS2, it was concluded that, indeed, this may be the case. This was an important observation, since the amount of

Table 4.1 Changes in total soluble nitrogen (TSN) and amino nitrogen (AN) during canola-mash fermentation (averages of 2 determinations).

Time (days)	TSN (% w/v)			AN (% w/v)		
	CS1	CS2	CS3	CS1	CS2	CS3
0	0.92	1.11	0.843	0.33	0.35	0.28
2	1.32	1.31	1.26	0.50	0.42	0.45
4	1.37	1.31	1.24	0.53	0.45	0.48
6	1.29	1.36	1.30	0.48	0.46	0.48
10	1.28	1.35	1.28	0.52	0.44	0.40
14	1.34	1.43	1.34	0.53	0.46	0.53
18	1.20	1.43	1.28	0.43	0.44	0.44
22	1.31	1.40	1.35	0.46	0.44	0.46
26	1.32	1.38	1.34	0.44	0.48	0.51
31	1.38	1.37	1.35	0.47	0.51	0.44

CS1: Canola sauce prepared by sequential or stepwise inoculation of *P. halophilus* and the yeasts (*S. rouxii* and *T. versatilis*).
CS2: Uninoculated canola sauce.
CS3: Canola sauce prepared by an all-in inoculation of *P. halophilus*, *S. rouxii* and *T. versatilis*.

TSN present in the sauce was considered an indication of quality and price of the product.

The fluctuations in TSN (Table 4.1) which occurred during the fermentation were also observed by Yong and Wood (1977b), and they attributed the decreases to periods when the number of microorganisms increased rapidly. A similar occurrence was observed with *P. halophilus* and yeasts in the canola mash.

It was evident from TSN value of the enzyme hydrolysate (EH) in Table 4.2 that most of the TSN was produced initially by the enzyme hydrolysis of the canola meal. This indicated an important step whereby the quality of the soluble nitrogen present in the final sauce could be maximized.

The amounts of AN reported in this experiment for the final canola sauces (Table 4.2) were low, ranging from 0.40% to 0.42%. However, during the fermentation process, the AN values ranged from 0.28% to 0.53%, with the sauces on day 31 containing 0.47, 0.51 and 0.44% for CS1, CS2 and CS3, respectively. It is thus apparent that refining the raw sauce reduced the AN content, possibly through the precipitation of some proteinaceous components. Ma (1985) reported values ranging from 0.66% to 0.80%, which are much higher than the values obtained in this experiment. The likely explanation for this discrepancy would seem to be the different methods of evaluation, and yet the value obtained in this experiment for commercial Kikkoman sauce (0.70%) was the same as was obtained by Ma (1985). Also, the initial AN obtained for the enzyme hydrolysate of 0.20% (Table 4.2) was much lower than 0.64% (Table 2.10) obtained by Ma. Although presence of microorganisms could cause a change in AN content (Yong and Wood, 1977b), there was no evidence to indicate that their involvement resulted in the lower values in this experiment. However, since the AOAC method (section 3.7.3.2) was applied in this experiment, while Ma (1985) adapted the method from Rosen (1957) for AN analysis, it could be suggested that the discrepancy was due to the different analytical methods used.

The quality of sauce depends on the ratio of AN to TSN, so that a value of 50% or more (Hesseltine and Wang, 1972) is regarded as good. The value obtained for the commercial Kikkoman sauce in this experiment was 53%. Ma (1985) quoted values of 48.50%

Table 4.2 Total soluble nitrogen (TSN), amino nitrogen (AN), nitrogen yield and AN/TSN ratio of refined canola and Kikkoman sauces (averages of 2 determinations).

Samples	TSN (% w/v)	AN (% w/v)	Nitrogen yield (%)	AN/TSN
CS1	1.20	0.41	67.19	0.34
CS2	1.21	0.42	67.75	0.35
CS3	1.20	0.40	67.19	0.33
KS	1.33	0.70	73.70[a]	0.53
EH	0.917	0.20	94.75[b]	0.22

EH - enzyme hydrolysate.
KS - Kikkoman sauce, produced commercially by Kikkoman Shoyu Co. Ltd., Japan.
Nitrogen yield - TSN in sauce/total nitrogen content in raw materials (4.465 g/100 g).
For an explanation of other abbreviations used, see Table 4.1.
[a] From Hesseltine and Wang (1972).
[b] Nitrogen content in 50 g Canola Meal = 3.05 g.

to 65.18% in canola sauces she produced (Table 2.10), these being higher than values obtained in this experiment of 34-35%. Factors such as raw materials, steaming conditions, koji starter and brine fermentation have been reported to affect AN:TSN ratio (Hesseltine and Wang, 1972). Thus, considering all of these factors, it is possible that the analytical methods for AN and perhaps the microorganisms involved during the fermentation caused the difference in the AN:TSN ratios.

Onaga *et al.* (1957) observed the importance of AN to quality of soy sauce. However, they also reported that a high percentage of AN was an indication of the acid-hydrolysis process, which they claimed differentiated soy sauce made by acid hydrolysis from that made by fermentation. Moreover, comparing 7 commercially prepared soy sauces, they reported that sauces with AN constituting only 31.4 and 32.5% of the total nitrogen (AN/TSN) were of better quality than sauces having 47.7 and 45.7%. This suggested that values reported in this experiment were quite acceptable and that the canola sauces were of good quality.

Nitrogen yield, another factor indicating quality of sauce, and expressing the efficiency of the enzymatic conversion of proteins contained in materials, was also evaluated. Hesseltine and Wang (1972) quoted a value of 73.70% as nitrogen yield in soy sauce, while Ma and Ooraikul (1986) recorded 61.34-73.38% in canola sauce (Table 2.10). Nitrogen yield of 67.19-67.75% was obtained in this experiment (Table 4.2), which falls within the experimental values reported in Table 2.10. Thus, the nitrogen yield for the canola sauce compared favourably with the literature value.

4.4.2.2 Amino acids in sauce product

It was apparent from the results obtained in this experiment (Table 4.3) that the amount of amino acids was far lower than was obtained by Ma (1985) (Table 2.11). Although the amount of amino acids in the commercial sauce used for comparison was also lower than found in Ma's experiment, there was the general observation that amino acids in canola sauce were lower than in commercial Kikkoman sauce. It was interesting to note that cystine, which was found in small quantities by Ma (1985), was not detected in any of the sauces. Perhaps the amounts were below the detection level in this experiment.

It was also observed (Table 4.3) that CS2 was lower in amino acids than CS1 and CS3. This indicates that the lactic and alcoholic fermentations proceeded with parallel conversion of more peptides to amino acids. Although serine was present in CS2, it did not separate well enough from threonine to be estimated.

The most important amino acid in soy sauce is glutamic acid, and results from experiment indicated that the canola sauces CS1 and CS3 contained greater quantities of the acid than the commercial sauce (Table 4.3). Udo (1931b), cited by Yokotsuka (1960), showed glutamic acid to be responsible for the delicious taste of soy sauce. Aspartic acid content was lower in CS1 and CS3 than in CS2 and Kikkoman shoyu. It is possible that fermentation with the added microorganisms was a factor in the reduction, which may partly account for the inferior taste of the canola sauces as compared to the commercial Kikkoman sauce.

Ma (1985) observed that small amounts of amino acid were produced during the enzyme hydrolysis of canola meal. Comparing amounts of amino acids in CS2 vs CS1 and CS3 (Table 4.3), it appears that about half of the total amino acids in the sauce was produced during the koji stage and the rest during the brine fermentation with the added microorganisms.

4.4.3 Acidic compounds in sauce product

4.4.3.1 Titratable acidity and organic acids

The total titratable acidity (TA) of the sauces (Table 4.5) varied between 6.75 to 10.19 meq NaOH/100 mL sauce. These values were much lower when compared to values reported by Ma (1985) (Table 2.12). This was because there was no adequate lactic acid fermentation in Ma's sauces. It is interesting to note, however, that the commercial Kikkoman sauce used in this experiment was found to contain a TA of 7.31 meq NaOH/100 mL, as compared to 33.35 meq NaOH/100 mL reported by Ma (Table 2.12). The discrepancy could be due to the sample preparation prior to titration. Ma (1985) passed sauce samples through both cation and anion exchange resins, to eliminate most basic components and used 4 M formic acid to elute the acidic fraction. No such treatment was given to the sauces in this

Table 4.3 Amino acid content in refined canola and Kikkoman sauces (μmole/mL, average of triplicate determinations).

Amino acid	CS1	CS2	CS3	KS
Aspartic acid	2.43	9.77	3.34	19.02
Threonine	10.10	5.92	9.41	14.60
Serine	15.27	UP	14.89	20.70
Glutamic acid	53.32	28.12	49.62	42.00
Proline	13.13	9.54	13.79	27.90
Glycine	16.66	11.34	16.00	19.75
Alanine	48.19	25.68	46.68	38.46
Cystine	--	--	--	--
Valine	18.15	12.36	17.90	29.84
Methionine	7.28	3.30	6.91	8.20
Isoleucine	13.21	9.06	13.21	25.12
Leucine	24.97	15.46	25.11	40.27
Tyrosine	6.64	5.03	6.42	3.03
Phenylalanine	8.74	6.95	8.82	16.09
Lysine	13.12	8.34	12.82	24.01
Histidine	3.70	1.33	3.08	3.36
Arginine	1.66	1.30	1.75	8.01

UP: under threonine peak - not separated.
For an explanation of other abbreviations used, see Table 4.1.

experiment, except decolourizing with charcoal. Therefore, the presence of certain components, perhaps both basic and neutral, helped reduce the total acidity. In fact, these values compare well with 6.13-19.97 meq/100 mL reported by Onaga *et al.* (1957) for seven commercial sauces analysed using a similar procedure but with neither pretreatment.

There was no correlation observed between the pH of sauces and their total acidity (Table 4.5). This was also observed by Onaga *et al.* (1957). For instance, a pH of 4.52 was equivalent to 10.22 meq/100 mL; pH 4.8 was equivalent to 16.15 meq/100 mL, while pH 4.57 was equivalent to 19.97 meq/100 mL. Onaga *et al.* (1957) also reported that sauces with higher total acidity values of 16.15 and 19.97 meq/100 mL were judged to be more acceptable. However, this was in direct contradiction with sensory results in this experiment, where canola sauce with the highest total acidity (CS1) was judged least acceptable, while Kikkoman sauce, with lower total acidity, was judged most acceptable.

Although no *P. halophilus* was inoculated into CS2, analysis revealed that, 4 d after fermenting the mash, some lactic acid was present. Since investigation did not reveal any *P. halophilus* in the mash at the end of fermentation, it appeared that the increasing content of lactic acid in CS2 could be due to certain enzymatic reactions, or activity of some acid producing contaminant from the koji stage. The amount of lactic acid produced in CS1 and CS3 mashes increased in a rather irregular fashion throughout the fermentation (Table 4.4). As much as 2.03 % w/v and 1.85% w/v of the acid were recorded on day 31 for CS1 and CS3, respectively. Athough the inoculum used was the same in CS1 and CS3, it was apparent that inoculating the mash with only *P. halophilus* to start the fermentation enabled the production of a larger amount of lactic acid. It was also apparent that the competition among the microorganisms in CS3 resulted in the production of less lactic acid. The lactic acid contents in the canola sauces after refining (Table 4.4) were much higher than the amount found in Kikkoman shoyu. Yokotsuka (1981) indicated that a value of 1-2% w/v of organic acid, mostly lactic acid, existed in good quality soy sauces. However, on analysing the commercial sauce, only 0.5% w/v lactic acid was found. The amount of lactic acid, 1.88% w/v in CS1 and 1.6% w/v in CS3, may have accounted for the sharp taste which the panelists commented on

Table 4.4 Changes in lactic acid (% w/v) content during canola-mash fermentation (average of two determinations).

Time (days)	CS1	CS2	CS3	KS
0	-	-		
2	0.05	-	0.38	
4	1.59	0.22	1.36	
6	1.93	0.23	1.67	
10	2.05	0.24	1.43	
14	1.88	0.33	1.89	
18	2.00	0.46	1.74	
22	1.62	0.66	1.81	
26	1.95	0.82	1.77	
31	2.03	0.92	1.85	
•	1.88	0.67	1.60	0.5

- : absent.
• : refined sauces.
KS: commercial Kikkoman soy sauce.
For an explanation of other abbreviations used, see Table 4.1.

Table 4.5 Total acidity (meq NaOH/100 mL sauce), pH and organic acid[a] (mg/100 mL sauce) content of refined canola and Kikkoman sauces.

	CS1	CS2	CS3	KS
Total acidity[*]	10.19	8.06	6.75	7.31
pH	4.60	5.26	4.77	4.74
Citric acid	25.75	102.6	55.5	38.5
Pyruvic acid	6.18	85.5	76.9	8.85
Malic acid	34	trace	6.92	26
Unknown 1	++	++	++	++
Unknown 2	++	++	++	++
Succinic acid	trace	trace	trace	trace
Lactic acid	1547.5	669	1542.8	335.6
Formic acid	48.14	43.94	70.0	63.7
Acetic acid	77.25	84.2	201	86.0
Propionic acid	32.75	10.2	25.6	3.54
Pyroglutamic acid	10.48	3.04	5.28	41.9

For an explanation of abbreviations used, see Table 4.1.
[a] : average of triplicate determinations.
[*] : average of two determinations.
++ : significant amount.

during sensory evaluation.

Yong and Wood (1976) detected some souring in their uninoculated mash, which was produced aseptically. However, the koji for CS2 was not produced aseptically and yet some lactic acid was detected during the brine fermentation. These observations suggest that lactic acid could be produced in mash without prior inoculation of lactic bacteria and, as long as pH dropped to 5, yeast could be inoculated for subsequent alcoholic fermentation. However, results for CS1 and CS3 indicated that the presence of *P. halophilus* not only produced lactic acid but helped accelerate the pH drop, for yeast fermentation.

The HPLC method of analysis detected two unidentifiable peaks of significant size in the organic acid profile of the sauces. None of the major organic acids present in soy sauce had retention times similar to those of the unknown peaks. The organic acids found in the sauces are shown in Table 4.5. Lactic acid analysed with HPLC was found to be the major acid in the canola sauces. In fact, lactic acid content in canola sauce was greater than that in Kikkoman shoyu. Similar results were obtained when the enzymatic method of analysis (Section 3.7.4.2) was used, but the actual quantities of lactic acid differed between the methods. The discrepancy may be due to the fact that the sauce sample for the enzymatic method was not fractionated, while the sample for HPLC analysis had been passed through ion-exchange columns to remove basic and neutral compounds.

Trace amounts of succinic acid were detected in all the sauces. Formic acid, although undesirable, was also detected. Though it characterizes a sauce produced by chemical hydrolysis (Fukushima, 1985), formic acid exists in naturally fermented sauce too, as was observed in the Kikkoman shoyu.

The canola sauces also contained some formic acid, with CS3, the 'all-in' inoculated sauce, having the largest amount. As was mentioned previously, the presence of the yeasts at the initial stage of fermentation, when pH was around 7, produced some acetic acid; it was thus not surprising to detect a greater amount of acetic acid in CS3 than in the other sauces (Table 4.5). Levulinic acid, the other undesirable acid, was not detected in the canola sauces or the commercial sauce. This acid had also been implicated (Fukushima, 1985) to be characteristic of chemical soy sauce. Not all of the organic acids observed in the literature (Table 2.8) were found in the sauces analysed. However, those found during this experiment in general had values greater than those cited by Yokotsuka (1986) (Table 2.8), except pyroglutamic acid, which was higher in the commercial Kikkoman sauce than the canola sauces (Table 4.5).

It was also observed that the amount of acids in each canola sauce was different from the others. Obviously, the combination of these acids in different proportions gave the sauces their characteristic organoleptic flavour and aroma. In general, however, the concentrations of organic acids obtained in this experiment were greater than those obtained by Ma and Ooraikul (1986) (Table 2.12). This difference could be due to both the different moromi fermentation procedures and the different methods of sample treatment prior to the analysis.

The concentrations of organic acids in Kikkoman sauce used in this experiment were higher than those reported by Yokotsuka (1986). Since both sauces were manufactured by the same company, the difference could be attributed mostly to the analytical methods applied.

Although Yokotsuka (1960) reported the major acids found in soy sauce to include acetic, lactic, succinic and phosphoric acids, the analytical method used in this experiment could not determine phosphoric acid. Experiments revealed that phosphoric acid could co-elute with citric acid. However, being an inorganic acid, its presence was not deemed important during the HPLC analysis for organic acids.

4.4.4 Sugar content in sauce product

4.4.4.1 Reducing and non-reducing sugars

Glucose is the major reducing sugar found in soy sauce. As a breakdown product of larger sugar molecules, glucose is converted to lactic acid by the bacteria, and to alcohol by the yeasts. Yokotsuka (1985) reported 3-5% w/v reducing sugars in soy sauce, most of which was present as glucose. Yong and Wood (1976) demonstrated that the glucose content in soy mash increased from the start of fermentation to the end, with a few decreases in between the period. A similar observation was made of the canola mash being fermented, except that the overall increase was not as great as that reported by Yong and Wood (1976).

The reducing sugar content was measured as residual glucose present in the canola sauces. Values obtained in CS1 and CS3 (Table 4.6) were much lower than in CS2. The involvement of *P. halophilus*, *S. rouxii* and *T. versatilis* in metabolizing the glucose accounted for the difference in residual glucose content between CS1, CS3 and CS2. Residual glucose in CS2 (Table 4.7) compared well with values obtained by Ma (Table 2.13). This was expected as there was inadequate lactic and yeast fermentation in the sauces prepared by Ma. The conversion of the residual glucose to the metabolites was observed to be more efficient in CS1, which involved the stepwise inoculation, than in CS3, which contained mixed cultures from the beginning. Perhaps, due to competition presented in the mashes by the organisms and also conditions required to establish themselves, the conversion of the glucose was less efficient and slower in CS3. This may account for the higher glucose content on day 31 in CS3 (Table 4.6).

Although literature reports have indicated 3-5% w/v glucose for soy sauce, the commercial sauce used for comparison contained only 1.17% w/v glucose. Values of 0.75 to 1.56% w/v residual glucose were found in canola sauces (Table 4.7). The enzymatic method adapted for the residual glucose determination could be a factor bringing about the difference between values measured in this experiment and literature values. Most methods measured other reducing sugars, in addition to glucose.

Sucrose was determined as the non-reducing sugar and the values ranged between 0.13 to 0.43% w/v in the canola sauces (Table 4.7). Sucrose content in CS2 was greater than in either CS1 or CS3, which indicated that sucrose breakdown in CS2 did not proceed at the same rate as in CS1 or CS3. However, the sucrose content of the commercial sauce was comparable to the amount in CS2. This observation revealed that the deliberate inoculation of the mash with organisms aided the hydrolysis of sucrose in the mash, even though part of it was used up, possibly through endogenous metabolism.

The sucrose content of the sauces produced by Ma (1985) ranged between 0.49 to 6.58%, which was greater than that obtained in this experiment. Nevertheless, the amount of

Table 4.6 Changes in reducing sugar (residual glucose, % w/v) content during canola-mash fermentation (average of two determinations).

Time (days)	CS1	CS2	CS3
0	0.67	0.34	0.48
2	0.95	0.89	0.85
4	0.99	1.52	0.87
6	1.54	3.14	0.86
10	1.72	3.83	0.80
14	0.99	5.74	0.83
18	0.98	6.69	1.10
22	0.92	6.35	1.20
26	0.88	7.43	1.48
31	0.81	7.63	1.61

For an explanation of abbreviations used, see Table 4.1.

Table 4.7 Reducing sugar (residual glucose) and non-reducing sugar (sucrose) contents in refined canola and Kikkoman sauces (average of two determinations).

Samples	Glucose (% w/v)	Sucrose (% w/v)
CS1	0.75	0.20
CS2	7.48	0.43
CS3	1.56	0.13
KS	1.17	0.39

For an explanation of abbreviations used, see Table 4.1.

0.43% w/v contained in CS2 was comparable to the results of one of the sauces in Table 2.13. None of the sauces presented in Table 2.13 and CS2 produced in this study was fermented with the inoculated microorganisms.

It is important to note that the different raw materials used for canola sauce and soy sauce production, as well as the different procedures used for fermentation, could have contributed to the differences in sucrose content of the sauces.

4.4.5 Alcohols

The production of ethanol during brine fermentation is accompanied by glycerol production, as has been indicated in the literature review. Studies have shown that both glycerol and ethanol are metabolic products of *S. rouxii* in a saline environment. Glycerol was also the hydrolytic product of lipids during sauce fermentation, as reported by Yokotsuka (1960). In this experiment (Table 4.9), 0.38 to 0.53% w/v of glycerol were observed in canola sauces CS1, CS2 and CS3, which were lower than that in Kikkoman shoyu. However, depending on the nature of the raw material, i.e. defatted vs whole beans, it was possible to compare the results for canola sauces with that in the literature.

Values between 1.0-1.5% w/v glycerol have been reported in the literature, and Yokotsuka (1960) attributed this range to the use of whole soy beans. Defatted soy beans resulted in 0.4-0.5% w/v glycerol in the sauce. Yokotsuka (1985) has also reported that in recent years more *S. rouxii* is used for fermentation, thereby producing more glycerol. Also, a mixture of defatted and whole beans has resulted in the production of more glycerol.

However, the low glycerol contents in CS1, CS2 and CS3 (Table 4.9) were comparable to values reported by Yokotsuka (1960) to be present in sauce produced from defatted soybean meal. On the other hand, the larger value of 1.59% w/v glycerol, observed in the commercial sauce, was suggestive of either whole soybeans or a large yeast inoculum or a mixture of whole and defatted soybeans being used to produce this sauce.

The sweetness imparted by glycerol to sauce is organoleptically detectable when the amount of glycerol present is greater than or equal to 0.5% (Yokotsuka, 1960). This may

Table 4.8 Changes in ethanol (% w/v) content during canola-mash fermentation (average of two determinations).

Time (days)	CS1	CS2	CS3
0	-	-	0.02
2	-	-	0.12
4	-	-	0.30
6	0.12	-	0.57
10	0.38	-	0.76
14	1.09	-	1.41
18	1.61	-	1.49
22	1.85	-	1.68
26	1.98	-	1.85
31	1.77	-	1.71

For an explanation of abbreviations used, see Table 4.1.
- : absent.

Table 4.9 Ethanol[*] (% w/v), glycerol[*] (% w/v) and 2-phenylethanol[a] (ppm) contents in refined canola and Kikkoman sauces.

Samples	Ethanol	Glycerol	Phenylethanol
CS1	1.60	0.53	56
CS2	-	0.38	31.8
CS3	1.50	0.51	48
KS	1.62	1.59	156

[*] : average of two determination..

[a] : average of four determinations.

For an explanation of abbreviations used, see Table 4.1.

have contributed to the greater sweetness detected in the commercial sauce, in the sensory evaluation.

Ethanol (Table 4.8) was not detected throughout the period of fermentation of CS2, yet glycerol was found in the final product. Since glycerol is also produced by fermenting yeasts in 18% NaCl medium, the amount present in CS2 cannot be attributed to yeast fermentation as no yeast was inoculated in CS2. This glycerol may have been produced by the hydrolysis of some lipids, contained in the canola meal, during the koji stage. This observation also suggests that the amount of glycerol produced in CS1 and CS3 was not only due to the yeast fermentation but also to lipid hydrolysis, which may have occurred during koji making.

A trace amount of ethanol was detected on day zero in CS3. This mash had been inoculated with *S. rouxii* and *T. versatilis*, which ferment sugars to ethanol. Considering that not much time elapsed between inoculation and sampling on day zero, the ethanol present could not have resulted from yeast fermentation. Also, since neither CS1 nor CS2 contained ethanol on day zero, it could be suggested that the ethanol observed must have been contained in the 3-day old active inoculum. Nonetheless, the ethanol content increased gradually in CS3, whereas CS1, which was inoculated with yeasts later, at pH 5, increased faster in ethanol content. Although yeast inoculum was the same in both CS1 and CS3, a more suitable condition for yeast fermentation was created by the stepwise inoculation in CS1, hence 1.77% w/v ethanol in CS1 as opposed to 1.71% w/v ethanol in CS3 on day 31.

The ethanol content in sauce is very important. Yokotsuka (1972) reported that alcohol content of sauce was rated highest as the chemical component which contributed most to the preference of soy sauce. Without the alcoholic fermentation, Wood (1982) contended that the typical soy sauce aroma did not develop. Ma (1985) pointed out that the canola sauce lacked the reminescence of the myriad aroma associated with soy sauce due to deficiencies in alcoholic as well as acid fermentation.

A typically 'fully brewed' soy sauce contains 1-2% (v/v) ethanol (Wood, 1982). As much as 1.5 to 1.6% w/v ethanol was produced in the canola sauces in this experiment (Table

4.9), which compared well to the 1.62% w/v value observed in Kikkoman shoyu, and to values in the literature.

Fukushima (1985) reported that 2-phenylethanol, in addition to 4-ethylguaiacol and 4-ethylphenol, were responsible for the matured aroma of soy sauce. This aromatic alcohol and the alkyl phenols are metabolic products of *Torulopsis* spp., and accumulated at the latter stage of fermentation. Fukushima (1985) reported the amount of phenylethanol (PEA) present in soy sauce as 3 ppm. On the other hand, Yokotsuka (1986) cited work done by Nunomura and Sasaki (1981) on eight kinds of shoyu which contained 3.71 to 10.25 ppm of 2-phenylethanol. Osaki *et al.* (1985) reported as much as 27.9 ppm in their soy sauce fermented by immobilized cells. These values are low when compared to values obtained in this experiment (Table 4.9). The PEA in the 20-times diluted sample seemed to agree with literature values; i.e., the commercial sauce contained 7.8 ppm, while the canola sauces had 1.59 to 2.8 ppm of PEA. However, as much as 31.8 to 156 ppm was detected in the undiluted sauces. The differences in data tabulated here and that found in the literature might be largely due to the different methods applied for evaluation. However, the results of analysis suggested that canola sauces fermented with microorganisms (CS1 and CS3) contained less PEA than the commercial sauce, but more than CS2.

It is therefore not surprising, that the canola sauces ranked lower in aroma during sensory evaluation. It is also important to note that CS2 did not have as much PEA as CS1 and CS3. This difference was due to the absence of *T. versatilis*. Nevertheless, the amount obtained in CS2 suggested that some PEA was formed during fermentation, even without *T. versatilis*.

4.4.6 Salt content of sauce product

The salt content of the canola sauces was in the range of 17.29 to 17.52%, as compared to 17.64% obtained for the commercial sauce (Table 4.10). Fukushima (1985) reported that salt in soy sauce varied from 16-19 g NaCl/100 mL of sauce.

Table 4.10 Salt content in refined canola and Kikkoman sauces (average of triplicate determinations).

Samples	mV	Molarity	% NaCl
CS1	74.3	0.0300	17.52
CS2	74.8	0.0296	17.29
CS3	74.4	0.0299	17.46
KS	74.1	0.0302	17.64

For an explanation of abbreviations used, see Table 4.1.

4.4.7 Colour

The initial colour of canola meal, which was yellowish-brown, contributed to the final sauce colour. This was observed (Table 4.11) when the 'b' values for colour measurement of the sauces were compared. The positive 'b' values indicated the extent of the yellow tint, in which case the canola sauces maintained some of their yellow colour, whereas the Kikkoman sauce did not have any. However, CS2 had less yellow tint than CS1 and CS3. The results also showed 'a' values ranging from 1.5 for Kikkoman shoyu and 2.3 to 2.7 for canola sauces, which indicated that canola sauces were more reddish than the commercial one. Ma (1985) did not observe similar results. In her experiment, the Kikkoman sauce appeared to be more reddish than the canola sauces. The 'L' values, ranging from 4.8 for Kikkoman shoyu and 6.3 to 7.5 for canola sauces, indicated that the commercial Kikkoman soy sauce was darker than the canola sauces.

About 50% of koikuchi soy sauce colour is formed during fermentation and aging of mash (Yokotsuka, 1986), while the other 50% occurs during pasteurization. The latter depends primarily on heat, resulting in the Maillard browning reaction between amino compounds and sugars, both hexoses and pentoses, present in the mash. The different conditions which may have been used in the commercial soy sauce production could explain the difference in colour. Also, use of different raw materials could result in the intrinsic colour difference, as well as the availability of amino compounds and the sugars for Maillard reactions.

4.5 Sensory Evaluation

Although the two sessions conducted for the sensory evaluation involved the same samples, statistical analyses performed on the scores given by the panelists varied in both sessions. In session 1, analysis of variance and Duncan's multiple range test for the colour and flavour attributes showed that the order of sauce preference was from CS2, CS3, CS1 and KS, with CS2 being the least preferred. Although there was no significant difference amongst the sauces at the 1% level, the 5% level indicates that CS1 and KS were significantly different in flavour from CS3 and CS2. The order of preference for aroma and the overall acceptance,

Table 4.11 Colour measurements of refined canola and Kikkoman sauces.

Samples	L	a	b
CS1	7.5	2.3	1.7
CS2	6.3	2.3	0.7
CS3	7.5	2.7	1.8
KS	4.8	1.5	-1.3

For an explanation of abbreviations used, see Table 4.1.

on the other hand, was from CS1, CS2, CS3 to KS, with KS being the most preferred. However, there was no significant difference amongst the sauces at either the 1 or 5% levels (Appendix 2).

In the second session, the order of preference for aroma was from CS2, CS1, CS3 to KS, and for the other attributes, from CS1, CS2, CS3 to KS, in each case KS being the most preferred (Appendix 2). KS ranked highest in all categories in both sessions. Whereas CS3 was the most preferred amongst the three canola sauces in session 2, for all attributes, CS1 was preferred over CS3 and CS2 for the colour and flavour attributes in session 1. However, CS3 was ranked highest over CS2 and CS1 for the aroma and overall acceptance in session 1 (Appendix 2).

Kikkoman soy sauce was used for comparison as the purpose of the research was to produce canola sauce with similar qualities to the Japanese type soy sauce. It was obvious from the average scores for all the attributes (Table 4.12) that the Kikkoman sauce was superior to the canola sauces. CS1 was the least preferred amongst the sauces, while CS3 was ranked the best of the canola sauces. CS2, which did not undergo any deliberate fermentation, was ranked higher than CS1, which was fermented.

The score for the overall acceptance of the canola sauces was an unexpected observation, since the deliberate fermentation utilized to produce CS1 was supposed to improve the quality of the sauce and therefore it should rank better than CS2. However, it could be that the higher acidity in CS1, pH 4.6, could have caused this unexpected low score. Also, comments from the panelists indicated that CS1 had a very sharp taste, which could only result from high acid content. CS2, CS3 and KS all had higher pH's than CS1. A conclusion may be drawn from the scores (Table 4.12) that, despite the necessary fermentation (CS1), excessive acid, which imparts a sharp taste to the sauce, would render it inferior in sensory score to a sauce with less acid even though it did not undergo alcoholic fermentation (CS2).

Despite the fact that the results in Table 4.12 indicated that KS was superior to CS3, CS2 and CS1, it was necessary to examine the reliability of the panelists in their assessment of

Table 4.12 Average scores of sensory evaluation on canola and Kikkoman sauces (2 sessions).

	CS1	CS2	CS3	KS
Colour	5.82	6.07	6.32	7.29
Flavour	5.25	5.64	5.86	7.21
Aroma	5.21	5.25	5.90	7.21
Overall Acceptance	5.50	5.82	5.90	7.43

For an explanation of abbreviations used, see Table 4.1.

the sauces. Results of analysis of variance performed on the panelists for the two sessions indicated that there was a highly significant disagreement amongst the judges in their preference for the sauces. Moreover, it is very important to indicate that the panelists evaluated the same sauce samples differently, such that the mean scores varied for the same samples in the two sessions conducted (Appendix 3). The inconsistency in the panelists' judgement of the samples does not permit a conclusive statement to be made on the sensory evaluation of the canola sauces. However, it was obvious that the acceptance of the sauce depended heavily on the panelists' sensory perception of the product. The panelists were Orientals and members of the Department of Food Science, in the University of Alberta.

Generally, the canola sauces produced by deliberate fermentation lacked the aroma reminiscent of soy sauce, probably because the amount of inoculum of *P. halophilus* used may have produced excess acid which could then have given the sauces the distinctive taste and aroma. In addition, it is important to consider that the sauces being compared were made from different raw materials, i.e. canola meal as opposed to soy bean, in which case the final products would be different in their aroma and flavour properties. Another difference would be the method of fermentation, i.e. deliberate as opposed to natural fermentation, which was used to produce the commercial soy sauce, as well as the duration of fermentation and pasteurization processes.

Most commercial soy sauces have been fortified with artificial flavouring agents, which impart characteristic aroma and taste to the product. It is possible that addition of such agents, for example monosodium glutamate, ethanol and caramel, for colour and taste may improve the organoleptic properties of the canola sauces. Nevertheless, it has been shown that, even though their taste and aroma are quite different, the canola sauces produced by this deliberate fermentation had chemical compositions similar to the commercial soy sauce. It is possible that, with proper balance of the microbial cultures inoculated in the moromi stage, and appropriate treatment of the sauce before bottling, as is done by commercial companies, qualities of canola sauce could be made comparable to that of the best soy sauce on the market.

5. GENERAL SUMMARY AND CONCLUSIONS

The investigation carried out in this research confirmed that it was possible to improve the organoleptic quality of canola sauce by involving microorganisms to deliberately ferment canola mash. The addition of the microorganisms, namely *P. halophilus*, *S. rouxii* and *T. versatilis*, effectively produced lactic acid, ethanol and some volatile compounds which had been lacking in the previous sauce. Moreover, the procedure used in inoculating the mashes showed that the best way of shortening the fermentation period was by inoculating the microorganisms in a stepwise fashion.

It was evident from the chemical composition of the sauces which had been deliberately fermented that the 'stepwise' procedure was superior in the nitrogen containing compounds to the 'all-in' procedure. This is an important observation since one of the aims of sauce production is to convert the crude protein into a more utilizable form, which could easily be digested. A better conversion of proteins implied a greater amount of digestible components that would be available for consumption. The reducing sugar content and amounts of most of the organic acids were much less in the sauce produced with the 'stepwise' procedure (CS1), than in CS3, the 'all-in' procedure. Generally, however, other important chemical components in CS1 were greater than in CS3. On the other hand, from results of sensory evaluation, the 'all-in' product (CS3) was rated better. It suggests then that, though the chemical components present in the 'all-in' (CS3) product were lower than in the 'stepwise' product (CS1), the initial presence of the mixed culture in CS3 may have exerted some synergistic effects such that its organoleptic quality was found to be superior. However, considering that the amount of inoculum used was the same in each case, it could be that the *P. halophilus*, being alone initially in CS1, utilized a great deal of sugar, thereby producing more acid and reducing both glucose and sucrose contents. Consequently, this imparted an undesirable sharp taste to the product.

Although statistical analysis showed inconsistency in judgement of the products by panelists, there was consensus that the canola sauces had similar aroma. While this may largely be due to the raw material used, it is also possible that the volatile components resulting from excessive bacterial fermentation could be another contributing factor. In spite of the favourable chemical composition of the canola sauces, their organoleptic quality still requires some modification. For example, it would be essential to reduce the amount of *P. halophilus* inolculum to as low as 10^1-10^3 CFU/g of mash to reduce the amount of lactic acid in the final product. The reduction in lactic inoculum may also enhance the yeast culture to produce more alcohol and more volatile components responsible for the mature aroma peculiar to soy sauce. Further aroma improvement, involving the addition of certain flavouring agents common to commercial sauces, may rid the canola sauce of the 'raw' smell.

This experiment has shown that the deliberate fermentation of canola mash improved the chemical characteristics of canola sauces. Although these sauces did not acquire all the characteristic taste and smell of soy sauce, the modified chemical characteristics were a step toward the improvement of canola sauce quality. With a few adjustments to inoculum amount and period of fermentation, a better balance of the taste and flavour components is possible. The stepwise inoculation procedure appeared to indicate that this improvement to the sensory characteristics could be done within a shorter fermentation period than the 'all-in' inoculated procedure. Moreover, with chemical composition more comparable to the commercial Kikkoman sauce, it could be suggested that the stepwise inoculation would be a better procedure to adopt for the production of canola sauce.

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7. APPENDICES

Appendix 1

Protease activity.

Expressed as XS unit and defined as: an enzyme, a solution of which containing 1.5 g per litre, which, under the stated experimental conditions produced a filtrate with an optical density of 0.500 when measured in a 10 mm path length cell, had a strength of 36 XS units per gram (Yong and Wood, 1977a).

α-amylase activity.

Expressed as X unit per gram and defined such that: 'the enzyme has an activity of one X unit per gram, when 25.0 mg of that preparation reacts on 1 g dry weight of starch in a total volume of 55 mL, a temperature of 30°C and a pH of 6.0, so that the achroic point is reached in 15 minutes'. X units per gram = 15/2tc, where t = time to the achroic point (min) and c = concentration of the extract in g per 100 mL. Achroic point is the time at which the optical density reached 0.80, using a 10 mm-path-length cell, and a wavelength of 617.5 nm (Yong and Wood, 1977a).

β-amylase or exoamylase activity.

Defined as: 'the amount of enzyme which produced 1.0 mg of reducing sugar calculated as glucose under the experimental conditions (Goel and Wood, 1978).

Cellulase activity.

Expressed by estimation of reducing sugar and defined by: 'that amount of enzyme which produced 1.0 mg of reducing sugar calculated as glucose under the experimental conditions' (Goel and Wood, 1978).

Amyloglucosidase activity.

Expressed in enzyme units and defined as: 'that amount of enzyme which produced 1.0 mg glucose in one minute under experimental conditions' (Aidoo *et al.*, 1981).

Maltase activity.

Expressed in enzyme units and defined as: that amount of enzyme which produced 1.0 mg glucose in one minute under experimental conditions (Aidoo *et al.*, 1981).

Appendix 2

Duncan's Test results for sensory evaluation of canola and Kikkoman sauces (2 Sessions)

Attribute	Session	1% Level	5% Level
Colour	1	CS2 CS3 CS1 KS	CS2 CS3 CS1 KS
	2	CS1 CS2 CS3 KS	CS1 CS2 CS3 KS
Flavour	1	CS2 CS3 CS1 KS	CS2 CS3 CS1 KS
	2	CS1 CS2 CS3 KS	CS1 CS2 CS3 KS
Aroma	1	CS1 CS2 CS3 KS	CS1 CS2 CS3 KS
	2	CS2 CS1 CS3 KS	CS2 CS1 CS3 KS
Overall Acceptance	1	CS1 CS2 CS3 KS	CS1 CS2 CS3 KS
	2	CS1 CS2 CS3 KS	CS1 CS2 CS3 KS

A line typed under any samples indicates no significant difference at the given percent level among the samples.

Appendix 3

Mean scores of sensory evaluation tests on canola and Kikkoman sauces (2 sessions)

			Samples		
Attribute	Session	CS1	CS2	CS3	KS
Colour	1	6.21	6.00	6.07	7.00
	2	5.43	6.14	6.57	7.57
Flavour	1	5.93	5.71	5.71	7.57
	2	4.57	5.57	6.00	6.86
Aroma	1	5.71	5.86	5.93	7.00
	2	4.71	4.64	5.86	7.43
Overall Acceptance	1	5.71	5.71	5.79	7.29
	2	5.29	5.93	6.00	7.57